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PRINCIPAL INVESTIGATOR: David N. Krag, M.D.

CONTRACTING ORGANIZATION: The University of Vermont
Burlington, Vermont 05405-0160

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David N. Krag, M.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The University of Vermont
Burlington, Vermont 05405-0160

E-MAIL:
dkrag@salus.uvm.edu

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13. ABSTRACT ***(Maximum 200 Words)***

The ultimate goal of the project described in the present report is to perform *in vivo* screening experiments of phage-displayed random peptides libraries (RPLs) in human breast cancer patients, in order to identify peptides that will home specifically to their tumor tissue. In future work, these breast tumor-homing peptides will be used to develop novel breast cancer therapeutics. The work described in this annual report includes:

1) Construction of five new RPLs. 2) Toxicity testing of intravenous injection of peptide-phage in animals, which is detailed in an enclosed IND application. The IND needs only minor additions for approval by the FDA. 3) Several *in vivo* screening experiments in mice with tumors. The *in vivo* screening experiments we have performed in mice, with essentially the same protocol proposed for human screening, as well as other toxicity testing experiments, provide evidence that *in vivo* screening with phage-displayed RPLs is safe. In addition, the *in vivo* screenings have identified several consensus amino acid sequences. An especially exciting result is that some of the peptides we have identified by *in vivo* screening are strikingly similar to peptides described by others as specific inhibitors of matrix metalloproteinases 2 and 9, which are promising tumor targets.

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Introduction

This report describes the first year of a research project designed to investigate the effectiveness of *in vivo* screening of phage-displayed random peptides libraries (RPLs) in cancer patients. The purpose of this research is to identify small peptides that bind specifically to breast tumor targets, which can ultimately be used to develop effective cancer therapeutics with high specificity for tumor cells and low toxicity to normal cells. Peptides identified by phage-display RPL technology can not only bind targets with high specificity, their small size is considerably more optimal for drug development than larger tumor-binding molecules such as antibodies. Phage-displayed RPLs have been used by many laboratories, including our own, to identify small specific ligands to many molecular targets, including tumor targets. An exciting report in Science, by Arap et al, in 1998 (Arap, Pasqualini et al. 1998), described the screening of phage-displayed RPLs in intact mice bearing tumors. Peptides were identified by this "*in vivo* screening" that bound specifically to tumor tissue. The tumor-homing peptides, conjugated to doxorubicin, were remarkably effective at reducing the tumor burden and increasing survival in a mouse model. The ultimate goal of the project described in the present report is to perform similar *in vivo* screening experiments in human breast cancer patients, in order to identify peptides that will home specifically to their tumor tissue. In future work, these breast tumor-homing peptides will be used to develop novel breast cancer therapeutics. The work described in this annual report includes:

1) Construction of five new RPLs, in addition to the original RPL described in the original grant proposal.
2) Toxicity testing of intravenous injection of peptide-phage in animals, which is detailed in an IND application (included in the Appendix). The IND needs only minor additions for approval by the FDA.
3) Several *in vivo* screening experiments in mice with tumors. The *in vivo* screening experiments we have performed in mice, with essentially the same protocol proposed for human screening, as well as other toxicity testing experiments, provide evidence that *in vivo* screening with phage-displayed RPLs is safe. In addition, the *in vivo* screenings have identified several consensus amino acid sequences. An especially exciting result is that some of the peptides we have identified by *in vivo* screening are strikingly similar to peptides described by others as specific inhibitors of matrix metalloproteinases 2 and 9, which are promising tumor targets.

Body

Task I. *Construct a large panel of random peptide libraries (months 1-14). For each system, five libraries will be constructed with disulfide-constrained loops ranging in size from 8-12 amino acids. Each system displays peptides in a different structural and/or spatial context.*

The original proposal for this work included a description of a phage-displayed RPL constructed in a fuse 5 system (Scott and Smith 1990). The original library displays nine amino acid peptides flanked by cysteine residues (CX_9C). The cysteines allow formation of a disulfide bonded cyclic peptide. During the first year of this project we have constructed another RPL in the fuse 5 system of the form ($X_4CX_{10}CX_4$). Work by other groups using phage-display RPL technology (Affymax, Genentech)(Wrighton, Farrell et al. 1996; Cwirla, Balasubramanian et al. 1997) has suggested that libraries of peptides with 3-4 random residues outside the disulfide loop are more effective at providing a source of ligands to certain targets. The complexity of our new fuse 5 library (i.e. the number of different peptides it contains) is 1.8×10^7, approximately the same size as our original library. In another project in the laboratory, this library has recently been used to identify non-phosphorylated peptides that bind specifically to the SH2 domain of Grb7, a potential breast tumor target, but do not bind at all to other highly homologous SH2 domains. This illustrates the powerful capability of phage-display RPL technology to provide highly specific peptide ligands for leads in drug development.

The new fuse 5 library was constructed essentially as described (Cwirla, Peters et al. 1990; Scott and Smith 1990; Oligino, Lung et al. 1997), with appropriately designed oligonucleotides.

We have also constructed four new libraries in a pVIII system, which displays several hundred copies of each peptide per phage particle, (the pIII system displays five copies of each peptide per particle). The vector and cloning protocols for the pVIII system that we are using was kindly provided to us by Affymax. The cloning methods used were essentially as described in the Affymax protocols. The pVIII libraries we

constructed were very large: one of them contained almost 10 billion different peptides. We have found that the quality of the pVIII libraries is not as high as the pIII libraries. Clones isolated from our pIII libraries almost always contain an insert of the correct size, while approximately half of clones isolated from pVIII libraries contain a correct insert. This difference is likely due to the fact that the pVIII library is constructed using a phagemid/helper phage system, while in the pIII system, only E. coli cells infected by a phage clone with an insert in frame can multiply. However, even with a loss of half of the complexity, the pVIII libraries are much larger than the pIII libraries- 50 to 200 times as large. Therefore the pVIII libraries may prove more effective as a ligand source for *in vivo* screening as, the larger the libraries, the more likely they are to contain a specific ligand to any given target. Because the pVIII system displays so many copies of each peptide, pVIII libraries may also be able to identify at least some binding ligands, even weak binders, due to avidity effects. On the other hand, by presenting fewer copies of each peptide, the pIII system may identify ligands with higher affinity, as avidity effects may not be as pronounced as in the pVIII system. However, the avidity vs. affinity advantages/disadvantages may actually be the opposite as described above depending on the presentation strategy. While the peptides are more numerous per particle in a pVIII system, they are *locally* more monovalent than in the pIII system, where the peptides are very close to each other at one end of the phage particle.

We now possess a vast number of different peptides- over 15 billion, which are available to us for *in vivo* screening. We will continue to construct new libraries, as described in the statement of work, as presentation of peptides within a large variety of different contexts will optimize our chance of obtaining a hit to any given target. This is especially important for *in vivo* screening, as not only is there little information available on the structure of the ligands we expect to identify, there is little structural information on the molecules we will most likely be targeting. The targets may be completely unknown and may be, in fact, revealed by *in vivo* screening.

The libraries we have constructed this year are listed below:

Library type	Peptide structure	Library complexity
pIII	$X_4CX_{10}CX_4$	1.80×10^7
pVIII	$X_4CX_{12}CX_4$	2.98×10^9
pVIII	$X_4CX_9CX_4$	9.40×10^9
pVIII	$X_3CX_9CX_3$	8.15×10^8
pVIII	$X_4CX_8CX_4$	2.59×10^9

Task II. *Establish the safety of intravenous (IV) administration of phage RPLs in human patients with breast cancer. (months 1-6) There are numerous descriptions in the literature of IV administration of E. coli phage into humans with no toxic reactions of any kind reported.*

Several conferences, including a Pre-IND meeting, have been held between our laboratory and the FDA in order to design the preclinical studies necessary to establish the safety of IV injection of phage RPLs in humans. We have completed these preclinical/toxicity testing studies and have submitted an IND application to the FDA (included in the Appendix). The design and results of these studies are described in detail in the IND application. Excerpts from the IND application are provided below to facilitate review. In summary, we have injected 31 mice, both normal and tumor-bearing, with a peptide-phage library preparation by tail vein injection. These injections have been with up to $10^{11\text{-}12}$ phage TU, which, we anticipate, is at least as much, if not more than the comparable amount we will need to inject into humans in order to identify peptides which bind specifically to tumor cells. All mice injected with phage appeared completely normal immediately after injection, and the mice retained a normal appearance until sacrifice for tissue analysis. The only observed toxicity was hepatitis in three FVB mice (out of a total of 31 mice injected with peptide-phage) in one of the studies. We only saw this hepatitis in FVB mice (another strain was injected with the same peptide-phage preparation and no toxicity was noted), and FVB mice in other peptide-phage injection studies did not show signs of hepatitis or other toxicity. Laboratory mice are susceptible to several types of hepatitis. It is possible that the hepatitis was preexisting in this group of mice, caused by an infectious agent. We performed a test for

the presence of two bacteria, known to cause hepatitis in mice, on a hepatitis-positive liver section. This test was negative. However, tests for these two causes of hepatitis are better performed by culture, not possible in our case, as live tissue was no longer available. In addition, hepatitis in mice is more commonly caused by viruses such as coronavirus. The most effective tests for hepatitis viruses in mice are serologic, and the animals were no longer available to collect blood samples when the hepatitis was detected. We examined organs of one untreated FVB, and no hepatitis was seen, however this mouse was from a different cage and batch of FVBs. If further toxicity tests are necessary, we will collect a blood sample from each mouse before any IV injections or other treatment, to provide a control tissue sample that we can test for evidence of preexisting hepatitis. The pathology of the observed hepatitis also suggests it may have been preexisting. In humans, viral hepatitis has a distinctive distribution that involves triads and small foci in the lobules. Small foci in the lobules, in greater numbers, would also be expected for a serious bacterial septic infection. In "chemical" hepatitis in humans, for example, a toxic response to a poison or drug, the hepatitis is more widespread and generally involves all lobules in a distinctive distribution following expected blood flow in the liver. The liver pathology noted in the three FVB mice did not appear to be due to a toxic phenomenon and appeared more similar to hepatitis caused by an infectious (viral or bacterial) agent. While it is not known what type of hepatitis, if any, might be caused by phage in eukaryotes, it is highly unlikely that they are truly "infectious" to any cell other than E. coli cells (see discussion in Section 5 of the IND application). Therefore, one would expect that if the particles did cause a hepatitis, it would manifest itself in a manner that would more resemble a "chemical" or general hepatitis. Nevertheless, as addressed in the IRB included in the IND application in the Appendix, all patients will be carefully monitored for possible liver toxicity.

As addressed in the Statement of Work, and described in the IRB, throughout the screening process patients will be carefully evaluated for other adverse reactions, such as allergic reactions. In addition, the level of anti-phage antibodies in each patient will be measured by ELISA, both prior to and post screening. Dr. Hans Ochs, a collaborator on this project, who routinely measures levels of anti-phage antibody to phage in patients, will assist us in the development of this ELISA assay.

IND excerpt:

Toxicology Integrated Summary

Four *in vivo* studies (Study I-IV) were designed and implemented to assess the toxicity of phage random peptide library (RPL) screening in a mouse model. The toxicity studies were designed to mimic as closely as possible the scheme that will be used in phase I clinical trials. A total of 31 mice were injected with 3 different preparations of peptide phage (naïve peptide-phage, peptide-phage amplified once from tumor (φ Amp1x), or phage amplified twice from tumor (φ Amp2x)), and were monitored daily for three days or three weeks after phage injection for signs of toxicity. The FDA has suggested that 3 day and 3 week timepoints for organ harvest would allow us to evaluate both acute and chronic toxicity of peptide-phage injections. (See individual study sections for details.) At the end point of each study, 10 organs were harvested from each mouse and subjected to three analyses: hematoxylin & eosin staining (H&E) to assess pathology; immunohistochemistry (IHC) to look for the presence of phage particles (or at least intact phage coat proteins), which are not necessarily infective; and phage titering to determine the number of infective phage remaining. A brief description of each study follows. Complete details for each study may be found within each study section.

- Study I was designed to assess toxicity in mice, either 3 days or 3 weeks, following a single IV injection of naïve peptide-phage. This study will evaluate whether any toxicity will result from a single IV injection of phage particles. The number of each peptide present at this stage (approximately 20 picograms) is so small that any toxicity which might result would most certainly be caused by the phage particles alone. (Refer to Diagram of Study Design: Study I in Study I section.)

- Study II was designed to assess toxicity in mice following a single injection of phage amplified from tumor. This study will evaluate whether any toxicity will result from peptide-phage particles enriched for tumor binding. Specifically, IV injection of two types of peptide-phage will be evaluated: peptide-phage isolated

from a tumor and amplified; and peptide-phage passaged through and amplified from tumors twice. This study will determine whether the peptides displayed on phage which have been passaged/enriched through an intact organism and its tumors, can cause toxicity not caused by the phage particles alone. The study will attempt to answer the following question: will peptide-phage amplified from host tissue be enriched for peptides that bind to normal host tissue in such a manner to cause toxicity? (Refer to Diagram of Study Design: Study II in Study II section.)

- Study III was designed to study toxicity in mice following three sequential IV injections of three different peptide-phage preparations: naïve peptide-phage library, peptide-phage amplified from a tumor in another mouse (φ Amp1x) and φ Amp1x passaged again through a second tumor (φ Amp2x). (Refer to Diagram of Study Design: Study III in Study III section.) As for Study IV, below, these studies were designed to test whether sequential injection of naive peptide-phage, then peptide-phage amplified from tumor tissue, is toxic. However, Study III circumvents the complication of technically difficult mouse surgeries and tests the toxicity of peptide-phage preparations directly.

- Study IV was designed to study toxicity in tumor bearing mice following three sequential IV injections of three different phage preparations in the same mouse: (1)naïve library, (2)phage amplified from a tumor excised from the same animal (φ Amp1x) after injection of naïve peptide-phage library, and (3)phage amplified from a second tumor excised from the same animal (φ Amp2x) after injection of φ Amp1x. Each injection occurred on separate days, in the order listed, followed 10 minutes later by excision of tumor. (Refer to Diagram of Study Design: Study IV in Study IV section.) The purpose of Study IV is to carry out *in vivo* screening in animals using a protocol which is nearly identical to the planned clinical protocol. Study IV examines the toxicity of the complete *in vivo* screening clinical protocol and also begins to examine efficacy, by determining whether the procedure can enrich for certain phage-displayed peptides, some of which may bind specifically to tumor tissue.

  Three strains of mice (acquired from Jackson Labs in Bar Harbor, Maine) were used for the four toxicity studies described above: FVB, BalbC, and MRL/MpJ-fas$_{LPR}$(MRL). The FVB and BalbC mice are normal strains that have intact immune systems. However, the MRL mice develop massive lymph node enlargement, or lymphoproliferative disease, beginning around 8 weeks of age. Because their lymph nodes become markedly enlarged (tumors) these mice were chosen to provide us with tumors targets for our *in vivo* screening. While these mice have the advantage of producing tumors for screening, they have the disadvantage of dying rather early (and somewhat unpredictably, 3-5 months in our experience) compared to other strains of mice. Therefore, it was difficult to determine which MRL mice to choose for study. Study mice needed to have at least three tumors, yet, ideally, had to be healthy enough to survive 3 surgeries and live to the 3-week endpoint (as in Study IV). The problem of animals bearing sizeable tumors lacking the longevity of normal animals is not unique to this model, however, and will likely be the case with any animal tumor model we utilize.

Overall findings: (Please refer to individual Study sections for complete detail.)

- **Survival**: Of the 31 phage-injected mice in our studies, all but one survived to the endpoint. The mouse that died before the study endpoint was one of three surgical mice (#3), which died while under general anesthesia following the removal of tumor during the second surgery. (Refer to the Appendix for Survival Surgery Protocol.)

  Note that there were 32 planned phage-injected mice. However, one of these (see Study II) died in a restraint while preparing the animal for injection.

- **Gross appearance**: All non-surgical mice appeared normal for the study duration as observed by activity level, appearance of coat, and posture. The mice that had surgeries to remove tumors (n=3) were somewhat less active the first day or two following each surgery. As discussed above, surgical mouse #3 died during the second surgery, therefore there are limited observations for this animal. (Refer to Study IV for more

information.) Additionally, the surgical mice chewed and pulled their sutures; Mouse #2 removed its sutures to the point of opening an incision. The incision was cleaned and antibiotic ointment applied daily until the wound healed. Surgical mice (#1 and #2), which were subjected to a complete phage screening, similar to the clinical protocol except with more extensive surgery and anesthesia, progressed well to the end of the study.

- **Weights**: Mice were weighed daily, with the exception of some weekend measurements that are noted in the study sections. While we have not performed a statistical analysis of the data, generally the mice appear to have either maintained or gained weight (apparently, compared to controls, consistent with normal growth) throughout the course of each study with the following exceptions. One of the two groups of mice in Study II (mice injected with φ Amp1x) dropped an average of 9.1% (n=7) of their body weight on the day following injection with phage, but recovered from this weight drop by the following day. The surgical mice (Study IV) dropped about 8% of their body weight in the days immediately following surgeries and stabilized. This is not surprising in light of the fact that they underwent general anesthesia 2-3 times in a short period of time, and had large tumors removed that accounted for up to 3% of the their body weight over the course of 3 surgeries. For example, surgical Mouse #2 in Study IV weighed 47.6 g on the first day of surgery, and had 3 tumors removed in 5 days weighing 600 mg, 403.6 mg, and 431.8 mg respectively.

- **Phage titers**: Phage titers were performed to determine the number of infective phage present in tissues. On the day of harvest, tissues samples from each of 9 organs and in some cases blood, were collected. The tissues were weighed prior to being homogenized and incubated with *E. coli* for titering. (The titering procedure is described in detail in the Appendix.)

  At three days, most blood and tissues were highly positive for infective.

  At three weeks following phage injection, regardless of preparation (naïve, φ Amp1x, or φ Amp2x) all tissues were negative for infective phage in all mice except one. Surgical mouse #2 had some phage detected in most tissues at three weeks, although much less than the number of phage typically detected in tissues three days after injection. The only tissue that did not have infective phage detected at three weeks in this mouse, interestingly, was tumor tissue.

  Of the two surgical mice that survived to the 3-week endpoint, one was injected with phage immediately before euthanasia for reasons detailed in Study IV, therefore the tissues were, as expected, positive for phage. Infective phage were found in all but the tumor tissue of the remaining surgery mouse (#2) at 3 weeks, although less than the amount of phage detected in tissues titered at 3 days.

  Blood clearance was similar to that observed by Ochs et al with ϕX174 phage: phage were detected in the blood at 24 hours, had diminished a great deal by 48 hours and were not detected at 72 hours. In addition, fecal samples from each cage of mice were completely negative. All titering was done with approximately one fourth of a complete organ, and could detect as little as one phage transducing particle.

Summary of Phage Titer Results[1]

Study	3 Day Harvest	3 Week Harvest
I	ND[2]	-
II	+[3]	-
III	ND	-
IV	ND	+[4] (n=1 for this assay)

[1] +/- denotes presence/absence of infective phage
[2] Not done
[3] All tissues positive except for: mouse #3 blood and liver, and mouse #2 spleen
[4] All tissues of surgical mouse #2 positive for phage except tumor

- **IHC**: We perform IHC on mouse organs harvested 10 minutes after injection of phage. Positive control tissue of organs obtained immediately after phage injection without perfusion, give clear staining of blood vessels with no staining in other parts of the tissue, while IHC of organs from a mouse not injected with phage was completely negative. Slides for IHC were prepared by the Histology Department at FAHC according to their protocol (see IND Appendix) using rabbit anti-M13 primary antibody (Sigma B7786, lot 038H4885) at a concentration of 0.73 µg/ml, a 1:10,000 dilution of the stock provided by our laboratory. Slides were evaluated by Dr. Don Weaver, the pathologist collaborating with us on this project. Most tissues were negative for phage particles at three weeks. See table below.

Summary of IHC Results[1]

Study	**3 Day Harvest**	**3 Week Harvest**
I	-/+[2] (n=4)	- (n=4)
II φ Amp1x	- (n=3)	- (n=4)
φ Amp2x	-/+[3] (n=4)	-/+[5] (n=4)
III	ND[4]	- (n=6)
IV	ND	- (n=1)

[1]Staining is graded using a -to +++ scale for immunoreactivity.
[2]Spleens had trace/+ staining. All other tissues were negative.
[3]Livers of 3 out of 4 mice had + staining.
Spleens of 2 out of 4 mice had +/++ staining.
One lymph node had ++ staining.
One kidney had +/++ staining.
All other tissues were negative.
[4]Not done, by design.
[5]One liver and one lymph node had + staining. All other tissues were negative.

- **H&E:** All tissues in all studies were found to be of normal histology for the given strain except for 3 out of 4 FVB livers in Study II. The project pathologist found some histological differences inherent to the strain of mouse after looking at control animals. Specifically, MRL mice typically have: (1)enlarged lymph nodes, (2)enlarged spleens with markedly expanded white pulp with lymphoproliferative disorder, and (3)glomerulonephritis in the kidney. BalbCs also had a strain specific steatosis of the liver. This was substantiated by looking at a control BalbC liver. Therefore, we have qualified our pathology results by referring to data as "normal for the given strain" throughout the body of this document. Additionally, it is normal for mice in general to have spleens with extramedullary hematopoiesis.

Summary of H&E Results[1]

Study	**3 Day Harvest**	**3 Week Harvest**
I	NL (n=5)	NL (n=5)
II φ Amp1x	NL (n=3)	NL (n=4)
φ Amp2x	2 livers with hepatitis, remaining tissues NL (n=4)	1 liver with hepatitis, 1 liver with lymphoid aggregates, remaining tissues NL (n=4)
III	ND[2]	NL (n=6)
IV	ND[2]	NL (n=2)

[1]NL denotes normal histology for the strain
[2]Not done, by design.

Summary of Results: Study I

- **Survival:** All mice survived to end point.
- **Gross appearance:** Activity, behavior, and appearance were observed to be normal in all mice for the duration of the study.
- **Weights:** All mouse weights appeared to be stable throughout the study. Measurements were not collected for weekend timepoints.
- **Phage Titers:** All tissue and blood titers for infective phage were negative at three weeks. The three day results were not obtained due to experimental error.
- **IHC:** Most tissues were negative for immunoreactivity, indicating the absence of phage particles. The only tissue positive for phage staining were the spleens of mice euthanized three days after peptide-phage injection.
- **H&E:** All tissues, both those harvested 3 days and 3 weeks following injection of naïve peptide phage, were determined to have normal histology.

Summary of Results: Study II

- **Survival:** All mice survived to end point, except for one that died in the restraint prior to injection. This mouse was not replaced in the study due to a lack of available FVB mice.
- **Gross appearance:** Activity, behavior, and appearance were observed to be normal in all mice for the duration of the study.
- **Weights:** The mice injected with φ Amp1x dropped an average of 9.1% (n=7) of their body weight on day 1 following injection but their weights had returned to baseline by day 2. The mice injected with φ Amp2x either maintained or gained weight throughout the remainder of the study.
- **Phage Titers:** Three days after phage injection, there were infective phage present in all of the tissues except for the blood and liver of mouse #3 and the spleen of mouse #2 (both of these mice were injected with φ Amp1x). No infective phage were detected in any of the tissues collected three weeks after injection with either φ Amp1x or φ Amp2x.
- **IHC:** All tissues were negative for phage staining three days following injection of φ Amp1x. Most tissues from mice injected with φ Amp2x were negative at three days with the exceptions of: 3 livers, 2 spleens, 1 lymph node, and 1 kidney. All tissues three weeks following phage injection were negative for phage particles except for the liver of mouse #16, and a lymph node of mouse #15, both from the φ Amp2x-injected group.
- **H&E:** All tissues examined were normal for the strain with the exceptions of: (1) hepatitis findings in the livers of 3 FVB mice, and (2) one FVB liver with lymphoid aggregates. Sections of liver from mice with hepatitis were subsequently stained with Steiner Stain to rule out Helicobacter or Clostridium infection. No bacteria were identified. Refer to the tables for additional information.

Summary of Results: Study III

- **Survival:** All mice survived to the end point.
- **Gross appearance:** Activity, behavior, and appearance were observed to be normal in all mice for the duration of the study.
- **Weights:** Mice weights appeared to either remain the same or increase (consistent with normal growth/control mouse) over the course of the study.
- **Phage Titers:** Blood was free of infective phage 11 days after the third and final injection of peptide-phage. No infective phage were detected in any of the tissues collected three weeks after the third injection of phage.
- **IHC:** All tissues were negative for phage three weeks following phage injection.
- **H&E:** All collected tissues were found to be of normal histology for the strain.

Summary of Results: Study IV

- **Survival:** Two out of three mice (#1 and #2) survived to the 3-week endpoint. The third mouse (#3), died while under general anesthesia following the removal of tumor during the second surgery. It is likely that this animal died from excessive halothane anesthesia, due to the difficulty of monitoring the breathing and heartbeat of such a small animal, while maintaining sterile surgical technique (draping). Furthermore, as discussed previously (see Integrated Summary), the general health of MRL tumor-bearing mice is less than optimal, which may further complicate the already difficult procedure of performing general anesthesia in mice. (Refer to the IND Appendix for Survival Surgery Protocol.)

- **Gross appearance:** The mice were somewhat less active the first day or two following each surgery. As discussed above, Surgical Mouse #3 died during the second surgery, therefore there are limited observations for this animal. Additionally, two of the surgical mice (#1 and #2) pulled and chewed their sutures; Mouse #2 removed its sutures to the point of opening an incision. The incision was cleaned, and antibiotic ointment applied daily until the wound healed. The surgical mice, which were subjected to a complete phage screening, similar to the clinical protocol except with more extensive surgery and anesthesia (#1 and #2), progressed well to the end of the study. With the exception of a few days of recovery time after each surgery, the animals appeared normal for the duration of the study.

- **Weights:** The weight of Mouse #1 was measured on days 6-24 after *in vivo* screening and indicated a fairly stable maintenance of body weight during this time.

  Mouse #2 lost 2.4 grams of body weight (5.0%) on the day following the initial surgery. After this initial loss, the weight stabilized until day 8. Mouse #2 lost 10.5% of its body weight over the next two days, but put on weight steadily through the remainder of the study, with a final weight within 5% of the starting weight.

  Mouse #3 lost 3.4 grams of body weight (7.5%) in the two days following the initial surgery. No further weight measurements were made as the animal died during the second surgery. (Please refer to graph and table for details.)

- **Phage Titers:** Mouse #1 was injected with phage just before organ harvest to provide positive IHC controls, and so did not provide valid three week titer results. Mouse #3 did not survive the second surgery. Therefore, the only valid titers in Study IV were from Mouse #2. In Mouse #2, most tissues were positive for infective phage three weeks after completion of the *in vivo* screening procedure, although less phage were present than is typically seen only three days after phage injection. It is interesting that only this mouse, which underwent a complete *in vivo* screening procedure, had positive tissue titers at three weeks. All other mice did not have any infective phage present in tissues three weeks after injection of peptide-phage.

- **IHC:** Based on our titering results, we had expected to see positive IHC results in Mouse #2. However, at the three-week endpoint, all tissues were negative for phage immunoreactivity in Mouse #2. This is not altogether unexpected, as titering is a very sensitive technique for detection of phage, and can detect as little as 1 phage transducing unit (TU). In addition, far less tissue is used in the IHC technique than in titering, and far more phage particles need to be present for a positive IHC signal. Another explanation for this result is that the phage positive by titering might have been present in blood components which were not adequately washed away by perfusion, but which were washed away during IHC slide processing.

  Mouse #1 was injected with phage immediately following removal of one tumor and prior to all other tissues being harvested to provide us with positive IHC controls. As expected, the tumor removed prior to phage injection was IHC negative for phage while all other tissues were IHC positive.

- **H&E:** All tissues were determined to have normal histology for the strain.

An IND application detailing the above toxicity studies has been submitted to and reviewed by the FDA. The FDA has requested some additional information and changes in protocol (letter provided in the Appendix). All of the information the FDA would like clarified can be easily provided, and the suggested protocol changes appear to be minor. Therefore, we plan to submit a revised protocol within one week. Depending on the time it

takes to obtain the other necessary approvals (described in the next section), patient studies could be started within the next few months.

Task III. *Identify specific tumor-binding phage by in vivo screening and characterize clones as done routinely with in vitro screening.*

No studies will be initiated in patients until our *in vivo* screening with peptide-phage IND application is approved by the FDA. In addition to FDA approval, approval from the following committees must also be obtained prior to initiation of the study in humans:

1) The University of Vermont Institutional Review Board (IRB)
2) The NCI sponsored Vermont Cancer Center Protocol Review Committee
3) The U.S. Army MRMC Human Subjects Research Review Board

Although we have not begun the human *in vivo* screening studies addressed in Task III, we have performed five *in vivo* screenings in animals. The FDA has informed us that while proof of "efficacy" is not essential in order to obtain an IND for a Phase I trial (the proposed injection of RPL phage in humans is considered a Phase I trial by the FDA), they have also advised us that our application would be strengthened considerably by some demonstration of potential efficacy. In our case, the "product", *in vivo* screening, is not really a drug- it is a process. The "efficacy" of this process is its ability to identify consensus amino acid sequences of peptides which specifically bind to tumor tissue. Therefore, we performed several *in vivo* RPL screening studies in mice with tumors, following essentially the same protocol planned for screenings of breast cancer patients.

We have located a valuable model for our studies: MRL mice, retired female breeders, which develop multiple spontaneous tumors. They develop 3 or more tumors, which are excisable with minor surgery. In addition, these animals are far easier to obtain and less expensive ($10 each) than transgenic mice which develop mammary tumors ($150 each). We received only a few transgenic mice almost a year after placing the order. However, we plan to start a breeding colony for the transgenic mice to test the efficacy of *in vivo* screening in two different *in vivo* cancer models. We have had some problems with premature death in the MRL mice with tumors, which can complicate toxicity and survival studies. However, this complication may be expected with any animal model where the animals bear palpable tumors, i.e. with animals that are sick with cancer.

We have performed five *in vivo* screenings. In the first *in vivo* screen, three sequential screens were performed in three different mice. This study was performed before we had successfully developed a method to remove endotoxin from the phage preparations, and so "survival surgery" experiments, where successive screenings are performed in the same animal, similar to the proposed human protocol, were not possible due to the high level of endotoxin in the phage preparations. This first screen identified several consensus sequences including a consensus sequence with high homology to a peptide MMP inhibitor(Koivunen, Arap et al. 1999).

After we developed a protocol to remove endotoxin from peptide-phage preparations to levels permitted by the FDA, the other four screenings were performed each in one mouse with multiple tumors, using a protocol very similar to that proposed for human studies. The original reports on *in vivo* screening in mice (Pasqualini and Ruoslahti 1996; Arap, Pasqualini et al. 1998; Rajotte, Arap et al. 1998) were performed by IV injection of RPL phage, followed by perfusion through the heart in order to wash non-binding phage from the tumor tissue, which, of course, killed the animals being screened. Obviously this procedure cannot be performed in humans. The washes must take place after the harvest of tumor tissue. Even with this modified screening and washing procedure, our *in vivo* screening resulted in the identification not only of consensus amino acid sequences, but sequences which may bind to an important tumor target.

The *in vivo* screening protocols used in our laboratory, including phage preparation, are described in detail in the IND application. In brief, the *in vivo* screenings we performed in mice consisted of a tail vein IV injection of a mouse that had three discrete tumors (approximately 0.2 g each) with a large dose of peptide-phage library. The first tumor was removed by minor surgery, under general anesthesia. The excision was sutured, and peptide-phage were amplified from the tumor tissue. The peptide-phage amplified from this first

tumor were purified, sterilized, and reinjected into the same animal the following day, and a second tumor was removed. Likewise, peptide-phage which bound to this second tumor were amplified, purified, and reinjected the next day for a third *in vivo* screen. A third tumor was removed, and peptide-phage clones eluted from this tumor tissue were subjected to DNA sequence analysis.

Three of the four mice that underwent this procedure appeared healthy immediately after the third injection and remained healthy at least one week after the procedure, even after three surgeries in a three day period. Although resection without entering a body cavity is technically considered "minor"surgery, the mouse procedure is considerably more difficult technically and traumatic to the animal than the human procedure: the tumors removed from these mice are almost 1% of the animal's weight and must be performed under general anesthesia. The human screenings will consist of only small biopsies under local anesthesia. Two mice appeared normal and healthy during the entire evaluation period, (for 21 days after the last surgery), at which time the animals were sacrificed in order to harvest organs for toxicity studies.

The two mice that did not survive the *in vivo* screening procedure are described below:

The first complete "survival surgery" screening was performed by a first year medical student with no previous surgical experience on live mice, with assistance from a veterinary assistant. Although the animal looked quite healthy up to a week after the surgery, after this time the animal became edematous and died within a few days. The cause of death could not be determined, but the surgical technique (lack of shaving the incision site, stapling of the wound rather than suturing the wound, as well as general sterile technique) is suspect. The next three in vivo screenings with surgeries were performed by the PI. While the PI also had no experience with mouse surgeries, he has a great deal of human surgical experience as well as some experience on animals larger than mice. The first two screenings went perfectly- the mice looked healthy throughout surgery and the observation period until they were sacrificed in order to examine their organs for evidence of toxicity. The fourth and last mouse, although it survived and appeared healthy during and after the first surgery, this mouse died under anesthesia during the second surgery. Administering and monitoring anesthesia in a mouse is technically difficult, and it is likely that this mouse died of an overdose of Halothane, rather than as a result of the peptide-phage injection. Whether this is the case will be better assessed as we gain experience with mouse surgeries. Alternatively, performing similar experiments in larger animals will help circumvent the technical difficulties inherent in performing surgery under general anesthesia on animals as small as mice. We have already initiated dialogue with two different veterinary groups concerning the possibility of performing *in vivo* screening on dogs with mammary tumors, which are fairly common.

Phage were isolated from all tumor harvests. We have sequenced the DNA from approximately 200 phage clones harvested from tumor tissues. The sequences are shown in the tables that follow. These data identify peptides that fall into several different consensus sequence patterns. These amino acid consensus sequences may represent peptides that bind specifically to tumor tissue. Cases where two or more different clones had an identical sequence is especially suggestive of specific binding to at least some target, as the chance of obtaining two identical clones by chance is 1 in 20 million (the library complexity is 20 million). The additional clones with amino acids that line-up with the duplicate consensus sequences supply more evidence that specific binding clones have been obtained, although the target is not known at this time.

Especially promising is that one of the peptides we isolated from tumor tissue is highly homologous (see table below) to a peptide motif very recently identified as an inhibitor of matrix metalloproteinases (MMPs) MMP-2 and MMP-9, which are promising tumor targets (Koivunen, Arap et al. 1999). Interestingly, this MMP inhibitor was identified by *in vitro* phage-display RPL screening with purified target by the same group which pioneered *in vivo* screening. Importantly, the MMP-inhibiting peptide has demonstrated promising anti-tumor activity *in vivo(Koivunen, Arap et al. 1999).* We are presently developing assays to test the homologous peptides we have identified to see if they also inhibit MMP activity, tumor growth, and metastasis. MMP protein is commercially available, which should greatly facilitate assay development. In addition, Lew Cantley at Harvard has agreed to assist us with these studies, as a post-doc in his lab is actively studying MMPs.

We believe these preliminary results are very exciting and lend tremendous support to our hypothesis that *in vivo* screening in humans can be used to generate more effective cancer therapeutics. Please note that these data are unpublished.

We have initiated development of assays to test the specificity of promising peptide-clones, i.e. clones which display a peptide related to consensus sequences identified from in vivo screenings. We have performed assays similar to those described in the original *in vivo* screening reports (Pasqualini and Ruoslahti 1996; Arap, Pasqualini et al. 1998; Rajotte, Arap et al. 1998)from the Burnham Institute, which consist of IV injection of peptide-phage clones, followed by perfusion of the animal through the heart, and titering the number of phage in the tumor and in the brain as a control organ. We also examined the same tissues, as did the Burnham group, by immunohistochemistry with an anti-phage antibody. Some of our initial titering experiments looked very promising and showed even greater selectivity for tumor compared to brain than the peptide-phage described by Arap et al (Arap, Pasqualini et al. 1998) (see table in Appendix). However, a recent experiment we performed with naive library phage, not expected to bind to any tissue, also demonstrated tumor selectivity. Articles on *in vivo* screening from the Burnham institute report very low binding of library phage to either tissue. It is possible that our tumor system, different from the xenograft system used in the work by Arap et al, does not allow adequate "washing" of the tumor tissue by perfusion due to altered blood supply. It is also possible that the tumors in our system bind phage non-specifically. This would not be altogether unexpected as the tumors in our model are essentially just huge lymph nodes, and other organs of the reticuloendothelial system, such as spleen and liver, are known to bind phage non-specifically. Another difference in our results, compared to the Burnham group, is that we obtain many more phage from tissues, as assessed by titering, even when the same number of phage are injected. Therefore, we may need to improve our perfusion/washing technique. A scientist from Harvard with a great deal of experience performing mouse perfusions was kind enough to visit our lab to train us in this technique. After this training session, our perfusions caused the liver to blanche readily, which led us to assume we were perfusing adequately. Even with what appeared to be proper perfusion, we still obtain over a hundred times more phage from tissues than the Burnham group after injecting the same number by the same route. On the other hand, although our IHC assay gives highly positive staining of tissues harvested from animals recently injected IV with phage, (this assay was developed as a necessary element of our toxicity studies), all of the clones we have tested so far are negative by IHC assay. Although these negative IHC results are compatible with adequate washing by perfusion, they are not compatible with the Burnham group's results, as they obtained positive IHC staining with much lower numbers of peptide-phage on the tissue as assessed by titering. We will repeat these assays with injections of smaller numbers of peptide-phage and greater perfusion volumes in an attempt to achieve an acceptable background level of library phage binding to tumor tissue. The assays reported by the Burnham group are not described in enough detail to allow exact duplication. One technical detail which may account for the difference in the titering assays by our group compared to the Burnham group is the speed of centrifugation of homogenized tumor tissue. We have been centrifuging at a high g-force in order to recover any membrane fragments released by homogenization. However, this may decrease the separation of phage contaminated blood cells from tumor tissue. We will repeat our assays with slower centrifugations at this step. However, this does not explain why our clones are negative by IHC. Either our clones do not bind to any of the tissues we have examined by IHC, or our IHC may need to be increased in sensitivity. While our positive controls stain intensely, there are billions of phage present our present positive controls and we may need to optimize the assay using positive controls with much lower numbers of phage. In addition, we will construct phage clones described by the Burnham group to bind to a certain tissue, such as brain or kidney, to use as positive controls in the development of these assays. Another technique which may increase the sensitivity of detection of peptide-phage binding by IHC is to follow the wash perfusion by a fix perfusion, to avoid loss of peptide-phage which may bind to tissues specifically but with low affinity.

We will also construct two tumor-homing phage clones described by Arap et al, to see if they specifically bind tumor tissue in our two animal models, which are different than the model they employed.

Although the hypothesis stated in the original proposal specifically predicts the effectiveness of in vivo RPL screening in humans, which we have not attempted yet, we have made significant progress in corroborating the effectiveness of in vivo screening in mice, demonstrated by the Burnham group in their original proof of principle experiments. We have made important modifications to their procedure in order to allow in vivo screening in humans, and have accumulated enough evidence that our modified procedure is safe

in mammals to allow us to proceed soon to human screenings. It is of interest that we identified peptides completely different the Burnham group, although several are very similar to known binders/inhibitors of promising tumor targets- matrix metalloproteinases 2 and 9.

Key Research Accomplishments

- Construction of five new phage-displayed random peptide libraries which contain over 15 billion different peptides.
- Design (assisted by the FDA) of comprehensive preclinical studies that evaluate the safety of *in vivo* screening with phage-display random peptide libraries in an animal model.
- Completion of preclinical (animal) studies designed to evaluate/predict the safety of performing *in vivo* screening with phage-display random peptide libraries in human cancer patients. With the exception of 3 cases of hepatitis out of 31 animals, (which may have been preexisting), there was little evidence of toxicity in these preclinical studies.
- Completion and submission of an Investigational New Drug application to the FDA.
- The IND has been reviewed by the FDA. The FDA has requested minor revisions.
- Several complete *in vivo* screenings have been completed in mice with tumors. The procedure does not seem to harm the animals, and consensus peptides have been identified from these screenings.
- Some of the consensus peptides we have identified from tumor tissue by *in vivo* screening in mice with tumors have high homology with peptides known to bind to and inhibit MMP2 and MMP9, promising tumor targets associated with metastasis.

Reportable Outcomes

- An IND application for *in vivo* screening with phage-display random peptide libraries in human cancer patients has been submitted.
- A patent application for the *in vivo* screening process in humans has been submitted.
- This work was presented at the NCI site visit to the Vermont Cancer Center at the University of Vermont. The NCI gave the VCC high scores and funding was continued.
- A manuscript describing our preclinical studies which demonstrate the safety of *in vivo* screening in animals is in preparation.
- A grant proposal that combines a concurrent project our laboratory is working on, the isolation of cancer cells from the blood of breast cancer patients, with human *in vivo* screening, is in preparation. In this project we propose isolating cancer cells from the blood of patients after injection of RPLs, in order to identify peptides which bind specifically to cancer cells which are found in the blood. Assuming that the entry of these cancer cells into the blood is one of the first stages in metastasis, the surface of these cells could be an important tumor target.

Conclusions

We have constructed five new phage-displayed random peptide libraries which contain over 15 billion different peptides. This represents a vast pool of potential ligands to any given tumor target. We will continue to construct more libraries in order to further increase the number of differently shaped molecules we have available as a resource for ligands during *in vivo* screening.

With assistance from the FDA, we have designed and completed comprehensive preclinical studies that evaluate the safety of *in vivo* screening with phage-display random peptide libraries in an animal model. With the exception of 3 cases of hepatitis out of 31 animals, (which may have been preexisting), there was little evidence of toxicity in these preclinical studies. An IND application for phage-display RPL screening in human

breast cancer patients has been submitted to and reviewed by the FDA. The FDA has requested minor revisions, which we are in the process of addressing. Demonstration of the safety of IV injection of filamentous peptide-phage is a critical step to our goal of performing *in vivo* screening in patients, in order to obtain in humans the exciting results obtained by Arap et al in mice, i.e. to ultimately develop novel and effective tumor-specific therapeutics for humans. There are several other areas of research which would benefit from the demonstration of the safety of IV injection of filamentous phage in humans, including the use of filamentous phage as an effective vaccine vehicle and also as a vehicle for gene therapy. Both of these applications have been explored in animals, and have showed considerable promise.

Several complete *in vivo* screenings have been completed in mice with tumors. The procedure does not seem to harm the animals, and consensus peptides have been identified from these screenings. Some of the technical difficulties in performing *in vivo* screenings in mice may be circumvented with a larger animal model. Not only does the procedure seem safe, at least as assessed in mice, it also appears to have efficacy, i.e. consensus peptides can be identified this way, which corroborates the findings of Arap et al., although we have not yet demonstrated that the peptides we identified bind specifically to tumor tissue. However, some of the consensus peptides we have identified from tumor tissue by *in vivo* screening in mice with tumors have high homology with peptides known to bind to and inhibit MMP2 and MMP9, promising tumor targets associated with metastasis. Small peptides which bind specifically to tumors are valuable lead compounds for drug development of cancer therapeutics. If any of the peptides we identified prove to bind to a tumor target, such as MMP, for example, this will open up a novel and powerful means of not only identifying potential therapeutics, but also of identifying promising tumor targets.

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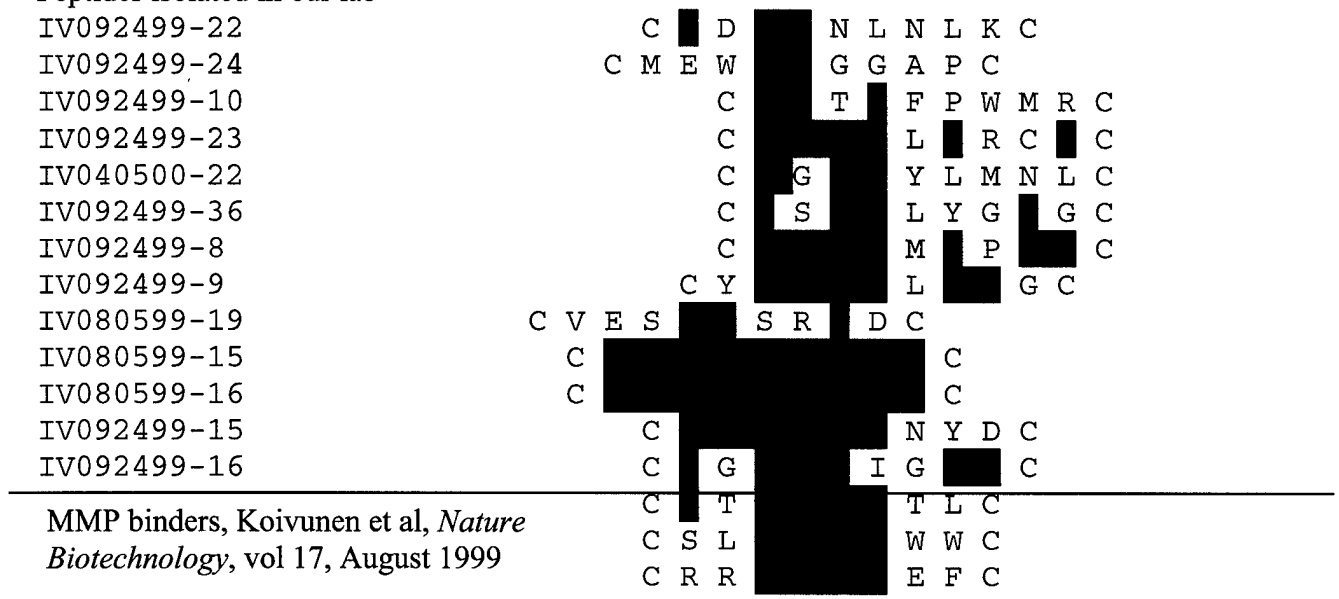
Similarity of peptides isolated in our lab by in vivo screening to MMP inhibitors

Peptides isolated in our lab



MMP binders, Koivunen et al, *Nature Biotechnology*, vol 17, August 1999

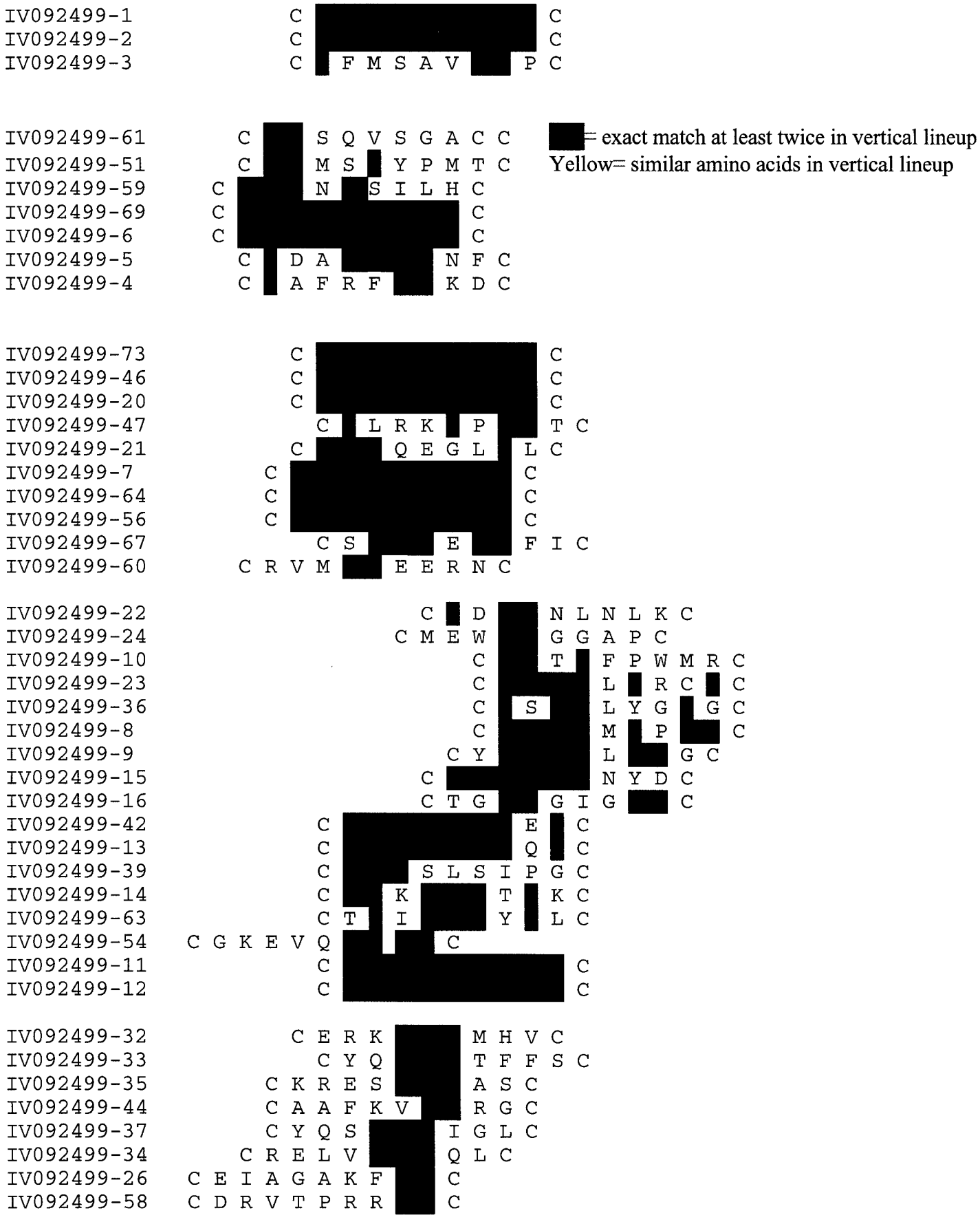
■ = exact match at least twice in vertical lineup
Yellow= similar amino acids in vertical lineup

Peptides isolated from the third tumor of the 8-5-99 in vivo screening

```
IV080599-15    C [redacted] C
IV080599-16    C [redacted] C
IV080599-19   C VES[redacted]SRVD C

IV080599-2     C RGSASA[redacted] C
IV080599-10         C G[redacted]VHPGN C

IV080599-11    C HGSL[redacted]PF C
IV080599-13    C MFKN[redacted]LK C

IV080599-5     C [redacted]TESMSRL C
IV080599-18    C [redacted]WDRVTIT C

IV080599-7     C DHAST[redacted]NG C
IV080599-12     C MEGG[redacted]INL C

IV080599-1     C RATSWWVTE C
IV080599-3     C DVFLVTDKL C
IV080599-4     C FSDCTSFNW C
IV080599-6     C YRADPSDPE C
IV080599-8     C KVSKAGEFN C
IV080599-9     C WKQGLTTSM C
IV080599-14    C YSGDNRLVI C
IV080599-17    C YPFTFLSPI C
IV080599-20    C VSGFHTIPG C
```

[redacted]= exact match at least twice in vertical lineup
Yellow= similar amino acids in vertical lineup

Peptides isolated from the third tumor of the 9-24-99 in vivo screening



IV092499-74	C	M	S	T	R	[redacted]	R	[redacted]		L	C					
IV092499-28					C		G			R	G	M	L	L	C	
IV092499-65				C	C		V			E	S	C	Y	C		
IV092499-29					C		K			G	T	A	K	F	C	

IV092499-17	C	Y	E	G	D	I	M	L	N	L	C
IV092499-18	C	T	L	N	R	N	G	F	R	H	C
IV092499-19	C	L	A	E	D	A	P	E	S	L	C
IV092499-41	C	E	L	L	S	G	Y	G	Y	L	C
IV092499-31	C	K	L	L	I	D	K	N	S	T	C
IV092499-25	C	V	D	F	N	M	I	S	I	N	C
IV092499-30	C	S	I	T	Q	V	G	M	Q	R	C
IV092499-38	C	P	E	Q	L	D	V	I	R	D	C
IV092499-40	C	G	L	Y	G	S	T	I	D	S	C
IV092499-43	C	A	H	N	D	R	S	L	T	L	C
IV092499-45	C	L	S	M	A	T	L	D	S	P	C
IV092499-48	C	V	F	R	F	G	A	V	G	I	C
IV092499-49	C	A	T	Y	F	D	Q	E	K	D	C
IV092499-50	C	S	V	G	L	E	K	T	R	A	C
IV092499-52	C	R	W	W	D	Q	G	E	N	H	C
IV092499-53	C	F	Y	K	S	V	K	R	L	M	C
IV092499-55	C	R	A	S	D	G	I	W	A	V	C
IV092499-57	C	W	N	S	L	W	A	T	R	I	C
IV092499-62	C	H	M	E	P	W	H	Q	V	C	C
IV092499-66	C	I	F	Y	R	N	R	V	F	C	
IV092499-68	C	H	E	H	Q	F	F	F	S	E	C
IV092499-70	C	S	S	D	L	Y	E	S	G	F	C
IV092499-71	C	G	M	F	T	S	E	L	S	A	C
IV092499-72	C	A	G	L	F	K	E	V	D	R	C

Peptides isolated from the 2-00 in vivo screening

IV020200-9 C [redacted] KLDINSC
IV020200-4 C YDP [redacted] TS C
IV020400-18 C [redacted] C
IV020400-41 C [redacted] C

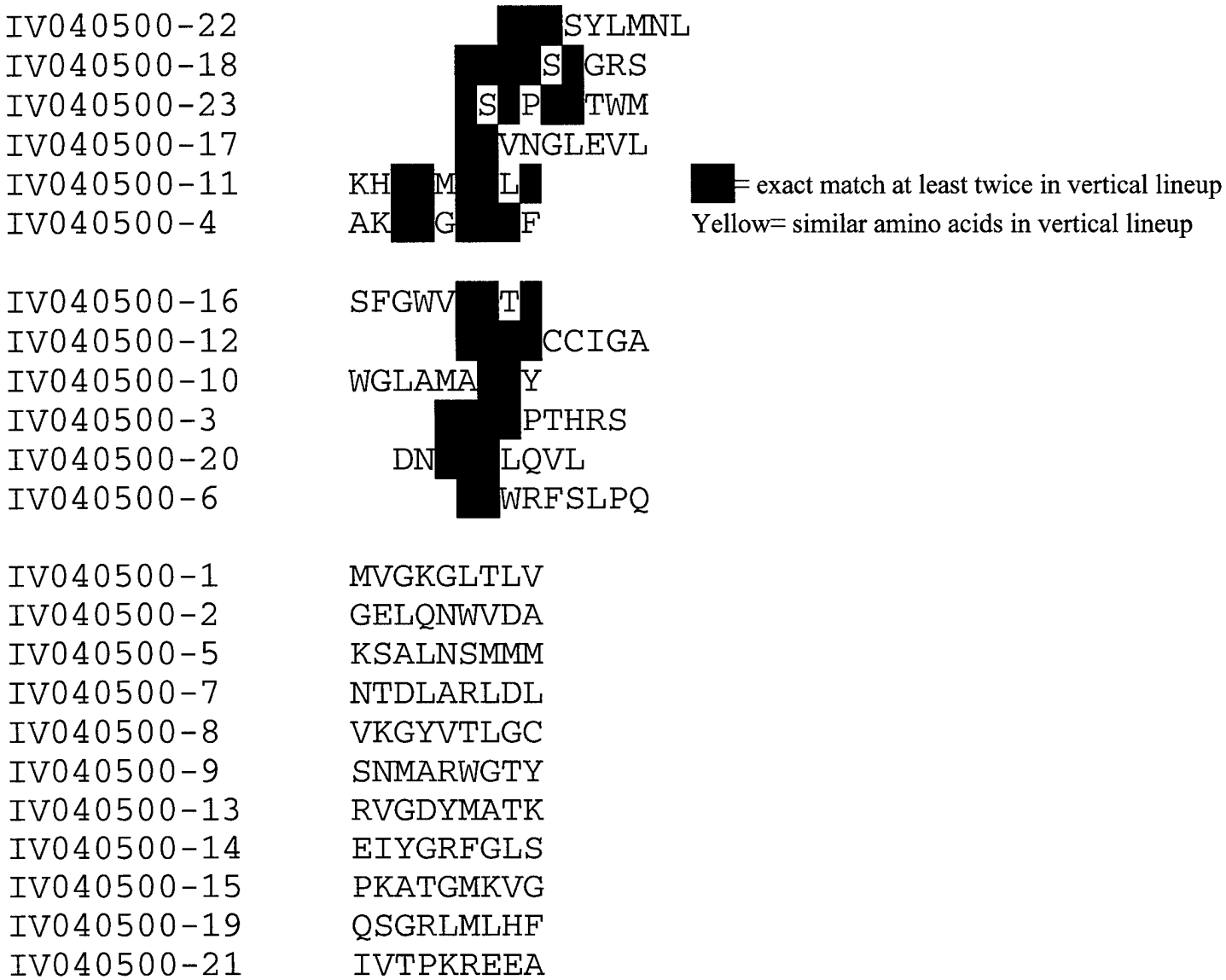
[redacted] = exact match at least twice in vertical lineup
Yellow= similar amino acids in vertical lineup

IV020400-44 C [redacted] ISE [redacted] A C
IV020400-14 C [redacted] HPS [redacted] S C

IV020400-13 C VTWGLYDVK C
IV020400-15 C GPQVVSEAL C
IV020400-16 C ITTNSNLTD C
IV020400-36 C SPLAQTTSS C
IV020400-17 C NEQGGALPK C
IV020400-24 C GSGGWYTD C
IV020400-34 C VAGEAGGPY C
IV020400-19 C QMADHSGPH C
IV020400-20 C MHTSGYIST C
IV020400-21 C IPDDEKLL C
IV020400-22 C RDSLVQSTM C
IV020400-23 C WWDVETRS C
IV020400-25 C MLPSSGLEQ C
IV020400-27 C EGLSSFTRS C
IV020400-45 C GYRSSVSVG C
IV020400-31 C SSSTMLEVV C
IV020400-26 C QKKGVRQLP C
IV020400-38 C MFMKKVRVM C
IV020400-28 C GRQGLVLEN C
IV020400-29 C VFETSGRHL C
IV020400-30 C KGSRVKDSG C
IV020400-32 C SPFLEETLQ C
IV020400-33 C NVKSNNEAA C
IV020400-35 C VGSALNSDV C
IV020400-37 C TAWDSLFDT C
IV020400-39 C SQVGITLRS C
IV020400-40 C DNFGARAHP C
IV020400-42 C AMYAPQNVF C
IV020400-43 C GKPVYGHIT C
IV020400-46 C YRGPDSWGM C
IV020400-47 C TRLGASDPL C
IV020400-48 C FLPTYKKDQ C
IV020400-49 C GPTPLEAGL C
IV020400-50 C TGRGNERFI C
IV020400-52 C SPSTAYISL C

IV020400-53	C	YQIPDMPKG	C
IV020400-54	C	VSYSMPPAL	C
IV020400-55	C	RFKIGAQVE	C
IV020400-56	C	GRHGDQGLD	C
IV020400-57	C	LRATEFYSV	C
IV020400-59	C	LTLSVHRAT	C
IV020400-58	C	ELFVKPVTA	C
IV020400-60	C	ATLEGVIKF	C

Peptides isolated from the second tumor of the 4-5-00 in vivo screening



Phage Recovered From Mice$_{MRL's}$ Tissues Following Perfusion

Clones Injected

IV092499-09	CYHMVSLENGC
IV092499-15	CWRHWVSNYDC
IV092499-16	CTGHWGIGENC
IV092499-20	CVLSDYIGGSC
IV080599-15	CSLWRHWPYIC

	01/12/00 Injected	01/12/00 TU/mg	07/05/00 Injected	07/05/00 TU/mg	07/27/00 Injected	07/27/00 TU/mg	08/09/00 Injected	08/09/00 TU/mg
IV092499-09	2.6×10^8	25.3_{tumor} 3.0_{brain} 8.8_{heart}	1.4×10^9	$3.9 \times 10^4{}_{tumor}$ $1.2 \times 10^4{}_{brain}$ $2.5 \times 10^4{}_{heart}$	2.3×10^9	$6.1 \times 10^4{}_{tumor}$ $5.1 \times 10^3, 4.8 \times 10^3{}_{brain}$ $TNTC_{liver}, >8 \times 10^4$	1.1×10^{10}	$1.0 \times 10^5{}_{tumor}$ $4.9 \times 10^3{}_{brain}$ $1.0 \times 10^4{}_{mammary}$
IV092499-15	1.8×10^7	$19.63, 3.7_{tumor}$ $6.5, 1.1_{brain}$ 2.9_{heart}						
IV092499-16	7.9×10^7	7.9_{tumor} 9.8_{brain} 6.7_{heart}						
IV092499-20			2.2×10^9	$3.6 \times 10^3, 6.1 \times 10^3{}_{tumor}$ $6.1 \times 10^2{}_{brain}$ $4.5 \times 10^3, 7.3 \times 10^3{}_{heart}$	5.9×10^9	$4.1 \times 10^3, 5.2 \times 10^3{}_{tumor}$ $1.4 \times 10^2, 2.4 \times 10^2{}_{brain}$ $TNTC_{liver}$	9.6×10^9	$4.2 \times 10^4{}_{tumor}$ $1.4 \times 10^3{}_{brain}$ $8.6 \times 10^3{}_{mammary}$
IV080599-15	7.5×10^6	7.0_{tumor} 1.2_{brain} 12.5_{heart}						
Naïve Library							7.0×10^9	$1.1 \times 10^5{}_{tumor}$ $3.6 \times 10^3{}_{brain}$ $NA_{mammary1}$

1. Tissue lost during aspiration following tissue homogenization and washing

Our Reference: BB-IND 9145

David N. Krag, M.D., FACS
Professor of Surgery
University of Vermont
309E Given Building
Burlington, VT 05405

Dear Dr. Krag:

Subsequent to our review of your **Investigational New Drug Application (IND)** for "Filamentous Bacteriophage Expressing Random Peptide Libraries," and as discussed during the telephone conversation of July 17, 2000 between you and Drs. David Essayan, Emmanuel Petricoin and Genevieve Schechter of this office, your proposed study under this IND has been placed on clinical hold.

Your IND is on clinical hold because insufficient information has been submitted to allow FDA to assess the risks to the subjects in the proposed clinical investigation [21 CFR 312.42(b)(1)(iv)]. Specifically, we have the following comments and requests for additional information:

PRODUCT INFORMATION

1. Research grade protease inhibitors should not be used to prepare the final drug product. This is especially so for leupeptin, which is derived from an uncharacterized bovine source. If the use of protease inhibitors is required for maintenance of phage diversity, the inhibitors should be obtained from BSE free sources. Certificates of analysis for each protease inhibitor must be submitted to the IND and should include information supporting freedom from potential adventitious agents and product impurities. Certificates of analysis should also be submitted to the IND for each lot of phage produced that supports sterility and freedom from product impurities.

CLINICAL INFORMATION

2. Plans for dose escalation and the number of patients to be treated at each dose level are not clearly delineated in Section 6.0 of the protocols. As discussed during the July 17, 2000, telephone conversation, you agreed to submit a revised study protocol as follows:

   The first patient will receive $10^{9\text{-}10}$ phage, and phage isolation from tumor will be evaluated. If no phages are isolated from the tumor, then you will treat a second patient with 10^{11} phages and attempt isolation from tumor. If the dose of phage must be escalated

to 10^{12} or greater, three patients must be treated at each dose level to assure safety of the intravenous injection of that concentration of phage. (The highest dose of intravenous phage that has been administered without toxicity reported in the literature is 2×10^{11} pfus.) Increase in the phage concentration must **not** be $> 10^{1}$ at any step in the dose escalation. Once an acceptable amount of phage is observed in the tumor specimen, amplification and readministration of phage to patients will be initiated with each patient to receive up to three injections over a period of approximately seven days. The concentration of phage used for reinfusions may not exceed the initial concentration used for that patient unless safety of the higher dose of phage infusion has been demonstrated previously in three patients.

3. You must incorporate in the treatment program (Section 6.0) of each program guidelines for the discontinuation of phage in the event of toxicity including the types/grade of toxicity that will result in discontinuation of phage injection. Injection of phage must be stopped immediately if Grade II or greater allergic reactions (NCI CTC Version 2.0) occur. Other NCI-CTC toxicities grade III or greater at any dose level are grounds for discontinuation of phage dose and for escalation of phage dose.

4. Reasons for discontinuation from study (Section 10) must be expanded. The phrase "constraints which are detrimental to the patient's health" is too broad. Toxicity such as anaphylaxis, other intolerable infusion related toxicities, or grade 3 or greater NCI –CTC toxicity (ies), other unexpected serious adverse events related to phage administration, patient refusal, or investigator decision due to a change in health status or non-compliance would be reasons for study discontinuation.

5. Specific criteria with regard to degree of organ (hematological, hepatic, renal, cardiac, pulmonary) dysfunction allowed for enrollment must be included in the eligibility criteria. The following guidelines are recommended:

   a. Hematologic function: Hgb $\geq$10 gm%, Hct $\geq$ 30%, ANC $\geq$ 1500/ul, Platelets $\geq$ 100,000/ul

   b. Renal: Creatinine < institutional upper limit of normal

   c. Hepatic function: Less than 2 x upper limit of normal; bilirubin < 2.0 gm%

   d. Cardiac: NYHA Grade II or less

   e. Pulmonary: no evidence of impaired lung function on physical examination; if evidence of pulmonary metastases or history of COPD or other pulmonary problem prior to enrollment must have FEV_1 and / or $DF_{CO} \geq 60\%$ for enrollment.

6. A subsection must be added to Section 11 to include information on the toxicity grading scale to be used to grade adverse event. Information about how the relationship of the toxicity to study drug will be reported should also be included in this section.

7. A statement must be incorporated to the Treatment Plan (Section 6.0) to indicate that patients will be evaluated approximately one month after completion of phage injection(s) for delayed toxicities.

8. Please submit revised copies of the two study protocols to the IND.

You may not initiate clinical trials under this IND until your response to the above deficiencies has been received and reviewed by FDA, and you are informed that the response is satisfactory. When you respond to all of the above issues, please identify your response as a "CLINICAL HOLD COMPLETE RESPONSE" and submit this information in triplicate to the IND. In addition, FAX a copy of the Form 1571, cover letter, and delivery tracking number to Lori Tull at 301-827-5397. For additional information, please refer to the FDA Guidance: Submitting and Reviewing Complete Responses to Clinical Holds - 5/14/98 (http://www.fda.gov/cber/gdlns/clinhold.pdf).

We also have the following comments and questions:

PRODUCT INFORMATION

9. Please provide information on the specificity, reproducibility, sensitivity, and quantitative reliability of the bacteriophage ELISA used for initial patient screening for previous exposure risks.

10. The current endotoxin administration limit for parenteral products is 5.0 EU/kg/dose or 5.0 EU/kg/hour. Please be advised that the proposed specification of 10 EU/kg/day for your product may limit your proposed clinical dose scheme. Are there batches of phages in which the extrapolated doses have exceeded that amount?

11. Please provide any available stability information on the phage stock, and describe the anticipated storage time and conditions. To support further product development, we recommend that a stability program be developed to establish storage conditions and a dating period for phage stock. The stability protocol should include an evaluation of product integrity and sterility and the specified time intervals for analysis.

12. In the pre-IND meeting held on December 16, 1999, it was agreed that 14-day sterility tests would be initiated, the results documented and appropriate actions undertaken in the event of a test failure, even though phage preparations would be re-administered prior to obtaining final test results. Please confirm.

13. Please quantitate levels of *E. coli* protein in the phage preparations, and set specifications.

14. Please provide certificates of analysis for the tritonX-114 and polyethylene glycol used in this study.

CLINICAL INFORMATION

15. You may wish to broaden the age requirements for both protocols to greater than or equal to 18 years of age.

16. The informed consent and protocol should be revised so that proposed period for phage administration is the same. The informed consent states a time period of 14 days while the protocol indicates a seven day time frame for phage injection. Please submit copies of the revised consent form that accurately reflects the proposed protocols.

17. If premedication is required to control acute toxicities related phage administration particularly with readministration, you should submit a protocol amendment.

18. Collection of information about the immune response to phage would be helpful for long term development. Please clarify your plans in this regard.

19. Reproductive status will be evaluated prior to enrollment and pregnancy testing performed when indicated. Male patients should be cautioned about unprotected intercourse. Please comment.

20. Estimation of the sample size should be revised to indicate that twenty patients will be studied once adequate phage isolation from tumor and amplification of the isolated phage occurs.

If you have any questions, please contact the Regulatory Project Manager, Lori Tull, at (301) 827-5101.

Sincerely yours,

Glen D. Jones, Ph.D.
Director
Division of Application Review and Policy
Office of Therapeutics
Research and Review
Center for Biologics
Evaluation and Research

1 Form FDA 1571

2 Form FDA 1572

3 Introductory Statement and General Investigational Plan

4 Protocols

5 Chemistry, Manufacturing and Control Data

6 Pharmacology and Toxicology Data

7 Previous Human Experience

8 Appendix

DEPARTMENT OF HEALTH AND HUMAN SERVICES
PUBLIC HEALTH SERVICE
FOOD AND DRUG ADMINISTRATION

INVESTIGATIONAL NEW DRUG APPLICATION (IND)

(TITLE 21, CODE OF FEDERAL REGULATIONS (CFR) PART 312)

Form Approved: OMB No. 0910-0014.
Expiration Date: September 30, 2002
See OMB Statement on Reverse.

NOTE: No drug may be shipped or clinical investigation begun until an IND for that investigation is in effect (21 CFR 312.40).

1. NAME OF SPONSOR
David N. Krag, MD, FACS

2. DATE OF SUBMISSION
6/19/00

3. ADDRESS *(Number, Street, City, State and Zip Code)*
University of Vermont, E309 Given
Burlington, VT 05405

4. TELEPHONE NUMBER *(Include Area Code)*
802-656-5830

5. NAME(S) OF DRUG *(Include all available names: Trade, Generic, Chemical, Code)*
Peptide Phage

6. IND NUMBER *(If previously assigned)*

7. INDICATION(S) *(Covered by this submission)*
Detection of short peptide ligands in patients with advanced cancer

8. PHASE(S) OF CLINICAL INVESTIGATION TO BE CONDUCTED:
☒ PHASE 1 ☐ PHASE 2 ☐ PHASE 3 ☐ OTHER _____ *(Specify)*

9. LIST NUMBERS OF ALL INVESTIGATIONAL NEW DRUG APPLICATIONS (21 CFR Part 312), NEW DRUG OR ANTIBIOTIC APPLICATIONS *(21 CFR Part 314)*, DRUG MASTER FILES *(21 CFR Part 314.420)*, AND PRODUCT LICENSE APPLICATIONS (21 CFR Part 601) REFERRED TO IN THIS APPLICATION.

10. ***IND submission should be consecutively numbered. The initial IND should be numbered "Serial number: 000." The next submission (e.g., amendment, report, or correspondence) should be numbered "Serial Number: 001." Subsequent submissions should be numbered consecutively in the order in which they are submitted.***

SERIAL NUMBER
000

11. THIS SUBMISSION CONTAINS THE FOLLOWING: *(Check all that apply)*

☒ INITIAL INVESTIGATIONAL NEW DRUG APPLICATION (IND) ☐ RESPONSE TO CLINICAL HOLD

PROTOCOL AMENDMENT(S):	INFORMATION AMENDMENT(S):	IND SAFETY REPORT(S):
☐ NEW PROTOCOL	☐ CHEMISTRY/MICROBIOLOGY	☐ INITIAL WRITTEN REPORT
☐ CHANGE IN PROTOCOL	☐ PHARMACOLOGY/TOXICOLOGY	☐ FOLLOW-UP TO A WRITTEN REPORT
☐ NEW INVESTIGATOR	☐ CLINICAL	

☐ RESPONSE TO FDA REQUEST FOR INFORMATION ☐ ANNUAL REPORT ☐ GENERAL CORRESPONDENCE

☐ REQUEST FOR REINSTATEMENT OF IND THAT IS WITHDRAWN, INACTIVATED, TERMINATED OR DISCONTINUED ☐ OTHER _____ *(Specify)*

CHECK ONLY IF APPLICABLE

JUSTIFICATION STATEMENT MUST BE SUBMITTED WITH APPLICATION FOR ANY CHECKED BELOW. REFER TO THE CITED CFR SECTION FOR FURTHER INFORMATION.

☐ TREATMENT IND 21 CFR 312.35(b) ☐ TREATMENT PROTOCOL 21 CFR 312.35(a) ☐ CHARGE REQUEST/NOTIFICATION 21 CFR 312.7(d)

FOR FDA USE ONLY

CDR/DBIND/DGD RECEIPT STAMP	DDR RECEIPT STAMP	DIVISION ASSIGNMENT:
		IND NUMBER ASSIGNED:

Created by: PSC Media Arts Branch (301) 443-2454 EF

12. **CONTENTS OF APPLICATION**

This application contains the following items: *(Check all that apply)*

- [X] 1. Form FDA 1571 *[21 CFR 312.23(a)(1)]*
- [X] 2. Table of Contents *[21 CFR 312.23(a)(2)]*
- [X] 3. Introductory statement *[21 CFR 312.23(a)(3)]*
- [X] 4. General Investigational plan *[21 CFR 312.23(a)(3)]*
- [ ] 5. Investigator's brochure *[21 CFR 312.23(a)(5)]*
- [X] 6. Protocol(s) *[21 CFR 312.23(a)(6)]*
  - [X] a. Study protocol(s) *[21 CFR 312.23(a)(6)]*
  - [X] b. Investigator data *[21 CFR 312.23(a)(6)(iii)(b)]* or completed Form(s) FDA 1572
  - [X] c. Facilities data *[21 CFR 312.23(a)(6)(iii)(b)]* or completed Form(s) FDA 1572
  - [X] d. Institutional Review Board data *[21 CFR 312.23(a)(6)(iii)(b)]* or completed Form(s) FDA 1572
- [X] 7. Chemistry, manufacturing, and control data *[21 CFR 312.23(a)(7)]*
  - [X] Environmental assessment or claim for exclusion *[21 CFR 312.23(a)(7)(iv)(e)]*
- [X] 8. Pharmacology and toxicology data *[21 CFR 312.23(a)(8)]*
- [X] 9. Previous human experience *[21 CFR 312.23(a)(9)]*
- [ ] 10. Additional information *[21 CFR 312.23(a)(10)]*

13. IS ANY PART OF THE CLINICAL STUDY TO BE CONDUCTED BY A CONTRACT RESEARCH ORGANIZATION? [ ] YES [X] NO

IF YES, WILL ANY SPONSOR OBLIGATIONS BE TRANSFERRED TO THE CONTRACT RESEARCH ORGANIZATION? [ ] YES [ ] NO

IF YES, ATTACH A STATEMENT CONTAINING THE NAME AND ADDRESS OF THE CONTRACT RESEARCH ORGANIZATION, IDENTIFICATION OF THE CLINICAL STUDY, AND A LISTING OF THE OBLIGATIONS TRANSFERRED.

14. NAME AND TITLE OF THE PERSON RESPONSIBLE FOR MONITORING THE CONDUCT AND PROGRESS OF THE CLINICAL INVESTIGATIONS

David N. Krag, MD, FACS
Professor of Surgery

15. NAME(S) AND TITLE(S) OF THE PERSON(S) RESPONSIBLE FOR REVIEW AND EVALUATION OF INFORMATION RELEVANT TO THE SAFETY OF THE DRUG

David N. Krag, MD, FACS
Professor of Surgery

I agree not to begin clinical investigations until 30 days after FDA's receipt of the IND unless I receive earlier notification by FDA that the studies may begin. I also agree not to begin or continue clinical investigations covered by the IND if those studies are placed on clinical hold. I agree that an Institutional Review Board (IRB) that complies with the requirements set fourth in 21 CFR Part 56 will be responsible for initial and continuing review and approval of each of the studies in the proposed clinical investigation. I agree to conduct the investigation in accordance with all other applicable regulatory requirements.

16. NAME OF SPONSOR OR SPONSOR'S AUTHORIZED REPRESENTATIVE	17. SIGNATURE OF SPONSOR OR SPONSOR'S AUTHORIZED REPRESENTATIVE	
David N. Krag, MD, FACS	(KRAG) David Krag MD	
18. ADDRESS *(Number, Street, City, State and Zip Code)*	19. TELEPHONE NUMBER *(Include Area Code)*	20. DATE
University of Vermont, 309E Given Building Burlington, VT 05405	802-656-5830	6/19/00

(WARNING: A willfully false statement is a criminal offense. U.S.C. Title 18, Sec. 1001.)

Public reporting burden for this collection of information is estimated to average 100 hours per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to:

Food and Drug Administration
CBER (HFM-99)
1401 Rockville Pike
Rockville, MD 20852-1448

Food and Drug Administration
CDER (HFD-94)
5516 Nicholson Lane
Kensington, MD 20895

"An agency may not conduct or sponsor, and a person is not required to respond to, a collection of information unless it displays a currently valid OMB control number."

Please **DO NOT RETURN** this application to this address.

DEPARTMENT OF HEALTH AND HUMAN SERVICES
PUBLIC HEALTH SERVICE
FOOD AND DRUG ADMINISTRATION

STATEMENT OF INVESTIGATOR

(TITLE 21, CODE OF FEDERAL REGULATIONS (CFR) PART 312)

(See instructions on reverse side.)

Form Approved: OMB No. 0910-0014.
Expiration Date: September 30, 2002.
See OMB Statement on Reverse.

NOTE: No investigator may participate in an investigation until he/she provides the sponsor with a completed, signed Statement of Investigator, Form FDA 1572 (21 CFR 312.53(c)).

1. NAME AND ADDRESS OF INVESTIGATOR

David N. Krag, MD, FACS
University of Vermont
Given Building E309
Burlington, VT 05405

2. EDUCATION, TRAINING, AND EXPERIENCE THAT QUALIFIES THE INVESTIGATOR AS AN EXPERT IN THE CLINICAL INVESTIGATION OF THE DRUG FOR THE USE UNDER INVESTIGATION. ONE OF THE FOLLOWING IS ATTACHED.

☒ CURRICULUM VITAE ☐ OTHER STATEMENT OF QUALIFICATIONS

3. NAME AND ADDRESS OF ANY MEDICAL SCHOOL, HOSPITAL OR OTHER RESEARCH FACILITY WHERE THE CLINICAL INVESTIGATION(S) WILL BE CONDUCTED.

University of Vermont
Given Building E309
Burlington, VT 05405

Fletcher Allen Health Care
Colchester Avenue
Burlington, VT 05401

4. NAME AND ADDRESS OF ANY CLINICAL LABORATORY FACILITIES TO BE USED IN THE STUDY.

University of Vermont
Fletcher Allen Health Care
Colchester Avenue
Burlington, VT 05401

5. NAME AND ADDRESS OF THE INSTITUTIONAL REVIEW BOARD (IRB) THAT IS RESPONSIBLE FOR REVIEW AND APPROVAL OF THE STUDY(IES).

University of Vermont
Committees on Human Research
231 Rowell Building
Burlington, VT 05405-0068

6. NAMES OF THE SUBINVESTIGATORS *(e.g., research fellows, residents, associates)* WHO WILL BE ASSISTING THE INVESTIGATOR IN THE CONDUCT OF THE INVESTIGATION(S).

Donald Weaver, MD, Pathologist

7. NAME AND CODE NUMBER, IF ANY, OF THE PROTOCOL(S) IN THE IND FOR THE STUDY(IES) TO BE CONDUCTED BY THE INVESTIGATOR.

Created by: FSC Media Arts Branch (301) 443-3454 EF

8. ATTACH THE FOLLOWING CLINICAL PROTOCOL INFORMATION:

[X] FOR PHASE 1 INVESTIGATIONS, A GENERAL OUTLINE OF THE PLANNED INVESTIGATION INCLUDING THE ESTIMATED DURATION OF THE STUDY AND THE MAXIMUM NUMBER OF SUBJECTS THAT WILL BE INVOLVED.

[ ] FOR PHASE 2 OR 3 INVESTIGATIONS, AN OUTLINE OF THE STUDY PROTOCOL INCLUDING AN APPROXIMATION OF THE NUMBER OF SUBJECTS TO BE TREATED WITH THE DRUG AND THE NUMBER TO BE EMPLOYED AS CONTROLS, IF ANY; THE CLINICAL USES TO BE INVESTIGATED; CHARACTERISTICS OF SUBJECTS BY AGE, SEX, AND CONDITION; THE KIND OF CLINICAL OBSERVATIONS AND LABORATORY TESTS TO BE CONDUCTED; THE ESTIMATED DURATION OF THE STUDY; AND COPIES OR A DESCRIPTION OF CASE REPORT FORMS TO BE USED.

9. COMMITMENTS:

I agree to conduct the study(ies) in accordance with the relevant, current protocol(s) and will only make changes in a protocol after notifying the sponsor, except when necessary to protect the safety, rights, or welfare of subjects.

I agree to personally conduct or supervise the described investigation(s).

I agree to inform any patients, or any persons used as controls, that the drugs are being used for investigational purposes and I will ensure that the requirements relating to obtaining informed consent in 21 CFR Part 50 and institutional review board (IRB) review and approval in 21 CFR Part 56 are met.

I agree to report to the sponsor adverse experiences that occur in the course of the investigation(s) in accordance with 21 CFR 312.64.

I have read and understand the information in the investigator's brochure, including the potential risks and side effects of the drug.

I agree to ensure that all associates, colleagues, and employees assisting in the conduct of the study(ies) are informed about their obligations in meeting the above commitments.

I agree to maintain adequate and accurate records in accordance with 21 CFR 312.62 and to make those records available for inspection in accordance with 21 CFR 312.68.

I will ensure that an IRB that complies with the requirements of 21 CFR Part 56 will be responsible for the initial and continuing review and approval of the clinical investigation. I also agree to promptly report to the IRB all changes in the research activity and all unanticipated problems involving risks to human subjects or others. Additionally, I will not make any changes in the research without IRB approval, except where necessary to eliminate apparent immediate hazards to human subjects.

I agree to comply with all other requirements regarding the obligations of clinical investigators and all other pertinent requirements in 21 CFR Part 312.

INSTRUCTIONS FOR COMPLETING FORM FDA 1572
STATEMENT OF INVESTIGATOR:

1. Complete all sections. Attach a separate page if additional space is needed.
2. Attach curriculum vitae or other statement of qualifications as described in Section 2.
3. Attach protocol outline as described in Section 8.
4. Sign and date below.
5. FORWARD THE COMPLETED FORM AND ATTACHMENTS TO THE SPONSOR. The sponsor will incorporate this information along with other technical data into an Investigational New Drug Application (IND).

10. SIGNATURE OF INVESTIGATOR	11. DATE
David Kragh	6/8/00

(WARNING: A willfully false statement is a criminal offense. U.S.C. Title 18, Sec. 1001.)

General Outline of Planned Investigation

Approximately 20 patients will be subjected in vivo screening in an attempt to identify peptides that home specifically to their tumor tissue. We estimate the duration of this project to be 12 to 24 months.

All procedures will be performed in the University of Vermont General Clinical Research Center (GCRC). The staff at the GCRC are expert in observation of patients during experimental protocols. Patients will be admitted to the GCRC. An intravenous line will be placed. Baseline vital signs will be determined before infusion of phage and every 15 minutes during infusion and for 2 hours after infusion. The patient will be under constant supervision and equipment, medications, and personnel capable of treating allergic reactions will be immediately available.

A phage displayed RPL pool will be prepared and tested according to FDA. Before phage injection, one sample of tumor tissue from the patient will be biopsied, snap frozen and sixty slides will be prepared for later testing of selected clones for tumor binding affinity. The library will be diluted in 250 ml saline and infused intravenously over 10 minutes into the patient. An appropriate dose will be one in which peptide-phage are isolated from small tumor biopsies, and preferably, one in which peptide-phage isolated from tumor tissue display homologous amino acid sequences. Small amounts of tumor tissue will be biopsied in the procedure room of the GCRC at time points of 10 minutes and 24 hours post infusion. It is intended that the biopsies be as small as possible on the order of 1.0 gram of tissue.

The tumor will be rinsed to remove blood, the tissue will be ground, and *E. coli* will be added to amplify phage. Phage will be eluted from tumor cells and amplified. Phage will be amplified and quantified by titering, with results available within 12 hours. If (enriched) phage are present, they will be prepared for initial infusion and will be reinjected as soon as possible (1 to 2 days). The harvest and amplification will be repeated a maximum of 2 times for a maximum of three screenings in one patient. Screening will ideally be completed in less than 7 days to avoid patient Ab response to phage. Throughout the screening process patients will be carefully evaluated for adverse reactions.

Peptides displayed by phage isolated from tumor tissue will be routinely analyzed for both consensus amino acid sequences and tumor-binding. Binding to normal tissue will be assessed ~~by immunohistology~~ on normal, quick frozen tumor tissue excised at the same time as the tumor biopsy and on a large panel of 32 different normal human tissues not from the protocol patient. Immunohisotochemistry with anti-transferrin receptor mAb will be used as a positive control to assure tissue and assay reliability.
Any consensus sequences identified from phage eluted specifically from the tumor tissue will be excellent candidates for tumor-specific peptides. Promising peptides will be synthesized and tested for tumor specificity.

DAVID N. KRAG, MD, FACS

CURRICULUM VITAE

PII Redacted

PERSONAL DATA

Name: [redacted] David N. Krag, MD, FACS

[redacted] [redacted]

ADDRESS

Professional:

Professor of Surgery
University of Vermont
Given Building E309
Burlington, VT 05405
Tel: 802-656-5830

[redacted] [redacted]

EDUCATION

University of the Pacific Stockton, CA	1977 BA	Chemistry
Loyola Stritch School of Medicine Maywood, IL	1980 MD	Medical School

POSTGRADUATE CLINICAL TRAINING

Yale University Medical Center New Haven, CT	1980-1982	Internship
University of California, Davis Sacramento, CA	1982-1986	Surgery Residency

POSTGRADUATE FELLOWSHIP TRAINING

John Wayne Cancer Center University of California, Davis Los Angeles, CA	1983-1984	Surg Oncol Research

BOARD CERTIFICATION

National Board of Medical Examiners	Diplomat	
American Board of Surgery (#042794)	Diplomat	1987
American Board of Surgery (#042794)	Recertification	1997

FELLOW

American College of Surgeons 1991-Present

LICENSURE

California State Medical License #G047327
Vermont State Medical License #042-0008329

CERTIFICATES

Advanced Cardiac Life Support	1985
Physician's Assistant Supervisor	1987
Fluoroscopy Supervisor and Operator Permit	1988
Breast Ultrasonography	1994

HONORS AND ACHIEVEMENTS

Deans Honor Roll University of Pacific 1975-1977

Laboratory Instructor: Organic Chemistry and General Biology, University of the Pacific: Position held while undergraduate 1977

Dept. of Health and Human Services: National Research Service Award (#CA09010), Surg Oncol Research Fellowship 1984

Visiting Research Fellow in laboratory of George Hahn, Ph.D. Stanford University 1984

Award for Research, The Upjohn Company - $5,000
For study of prostaglandin as a mediator for hyperthermic destruction of cancer cells 1985

Award for Research, The Upjohn Company - $18,000
For study of the synergism between ARA-C and cis-Platinum 1985

American Cancer Society Career Development Award - $90,000 1987-1990

James E. DeMeules Research Award
Department of Surgery, University of Vermont 1994

APPOINTMENTS HELD

Assistant Professor of Surgery 1986-1991
School of Medicine
University of California, Davis

Assistant Professor of Surgery School of Veterinary Medicine (Joint appointment) University of California, Davis	1987-1991
Assistant Professor of Surgery University of Vermont Burlington, VT	1991-1993
Director, University of Vermont Breast Care Center Burlington, VT	1992-1995
Chief, Surg Oncol Service University of Vermont Burlington, VT	1992-Present
Associate Professor of Surgery University of Vermont Burlington, VT	1993-1998
Professor of Surgery University of Vermont Burlington, VT	1998
Graduate Group in Physiology University of California, Davis	1987-1991
Graduate College, University of Vermont	1991-Present

SOCIETIES AND MEMBERSHIP

American Association for Cancer Research
American College of Surgeons
American Institute of Ultrasound in Medicine
American Peptide Society
Association for Academic Surgery
Cancer and Leukemia Group B
John H. Davis Society
National Surgical Adjuvant Breast and Bowel Project
New England Cancer Society
Society of Surgical Oncology
Vermont Cancer Center

PROFESSIONAL SOCIETY COMMITTEES

National Surgical Adjuvant Breast and Bowel Project Principal Investigator for clinical trials, University of Vermont	1991-Present
National Surgical Adjuvant Breast and Bowel Project	1991-Present
Protocol Chair: Minimal Access Surgical Staging of Breast Cancer	1998
Society of Surgical Oncology Member Education Committee	1992
Cancer and Leukemia Group B Principal Investigator for clinical trials, University of Vermont	1994-1996
Board of Directors Cancer and Leukemia Group B	1994-1996
Member Core Breast Committee CALGB	1995-1997
American College of Surgeons Clinical Oncology Group:Protocol Chair	1997

for clinical trial, "The Sentinel Node in Elderly Breast Cancer Patients"

UNIVERSITY COMMITTEES

Search Committee for Chair of Medical Oncology	Member 1991-1993
Search Committee for Director of Vermont Comprehensive Cancer Center	Member 1991-1993
Graduate College UC Davis	Member 1985-1991
University of Vermont	Member 1991-Present
UC Davis Cancer Center	Member 1985-1991
Vermont Comprehensive Cancer Center (VCC)	Member 1991-Present
Clinical Oncology Advisory Committee of the Vermont Comprehensive Cancer Center	Member 1992-1998
VCC Clinical Research Protocol Review Committee	Member 1992-1994
Interviewer, VCC candidates for Associate Director of Clinical Affairs	1994
Interviewer, Gynecology/Oncology candidate	1994
VCC Clinical Cancer Services Task Group	1995-1998
VCC Surgical Coordinator, Surgery Core Committee, CALGB	1996
The Graduate College Executive Committee	1992-Present

HOSPITAL COMMITTEES

UC Davis Medical Center Medical Staff	1985-1991
Medical Center Hospital of Vermont	
Medical Staff	1991-present
Cancer Committee	1992-present
General Tumor Board Member	1991-present
Community Rounds Internship Program	Participant 1993
Committee on Utilization of Stereotactic Breast Biopsy	Member 1993
Breast Mass/Cancer Team	Member 1992-1996
Multidisciplinary Breast Conference	Chair 1992-1995
Academic Medical Center Committee on Quality Improvement Project for Breast Cancer (QIP)	Member 1994-1997

DEPARTMENT OF SURGERY COMMITTEES

Ad Hoc Committee/Call Schedule	Member 1994
Surgical Associates business meetings	Member 1991-present
Ultrasound Standards and Credentials Committee	Member 1992-Present
Residency Review Site Visit	Participant 1994
Proctor, Provisional appointment, Dr. Sheryl Peterson	1994
Proctor, Dr. Seth Harlow	1994

OTHER PROFESSIONAL ACTIVITIES

American Cancer Society, Vermont Division Board of Directors	1993-1996
Vermont Cancer Coalition/Professional Education/ Quality Assurance Committee	Member 1992-1996
Breast Cancer Professional Education/Quality Assurance Committee	Member 1993-1996
Cancer Liaison Officer, Medical Center Hospital of Vermont	1992-1995
American College of Surgeons Commission on Cancer, State Chair, Vermont Cancer Liaison Physicians	1995-1997
American College of Surgeons Commission on Cancer Vermont Tumor Registry Task Force	Member 1994
Lake Champlain Cancer Research Organization Scientific Advisory Board	1995-Present
Reviewer, Journal of Trauma	1994
Reviewer, Int J Hyperther	1994
Reviewer, Journal of the American Academy of Dermatology	1996
Reviewer, Archives of Surgery	1995/1998
Reviewer, Surgery	1998
Scientific Reviewer, Department of Defense grant	1998
Scientific Reviewer, California Breast Cancer Research Program	1998
Reviewer, Dutch Cancer Society, Amsterdam	1998
Reviewer, Kluwer Academic Publishers, Norwell MA	1999
Reviewer, CANCER, West Orange NJ	1999
Reviewer, Henry Ford Health Sciences Center, Detroit MI	1999
Reviewer, Journal of Clinical Oncology	1999
Reviewer, The Lancet, England	1999
Reviewer, Cancer, West Orange, NJ	1999
Reviewer, N Engl J Med, Boston MA	2000
Reviewer, Kluwer Academic Publishers, Norwell MA	2000
Reviewer, J. American Academy of Dermatology, Worcester MA	2000
Reviewer, Cancer, West Orange, NJ	2000
Reviewer, Breast Cancer Research and Treatment/Kluwer, Norwell MA	2000

POSTGRADUATE SEMINARS AND WORKSHOPS

Co-chairman-Biology Session, North American Hyperthermia Group Annual Meeting		1985
Director-	Ultrasonic Liver Surgery Workshop University of California, Davis	1989
Moderator-	Frontiers in Nuclear Medicine Symposium American College of Nuclear Physicians and U.S. Department of Energy, Washington, DC	1989
Director-	Gamma Probes for Intraoperative Detection of Radiolabeled Tissue, University of Vermont	1993
Director-	Gamma Probes for Intraoperative Detection of Radiolabeled Tissue, University of Vermont	1994

Chair -	Workshops for melanoma/breast staging for lymphatic resection protocols, American College of Surgeons convention, New Orleans, LA	1995
Chair -	Workshop for breast /melanoma lymphoscintigraphy studies Soc. of Surg Oncol, Atlanta, GA	1996
Participant-	Workshops (2) on ultrasound-guided biopsy on phantoms American College of Surgeons convention, San Francisco, CA	1996
Chair -	Investigators' workshop on melanoma trials Soc. of Surg Oncol, Chicago, IL	1997
Co-Chair -	Investigators' workshop for Sunbelt melanoma Society of Surgical Oncology, San Diego, CA	1998
Participant-	Minimally Invasive Techniques in Breast Care: A Workshop for Surgeons	1998
Instructor-	Annual Ultrasound for Surgeons & Surgical Residents Course, University of Vermont	1996-1999
Faculty-	New methods of staging breast cancer: University of Vermont Continuing Medical Education Program, Medical Alumni Reunion '99	1999
Faculty-	Premier One National Oncology Forum	1999

UNDERGRADUATE RESEARCH TEACHING

Nicholas Makhoul Awardee, Presidents Undergraduate Fellowship Award	1988
Uy Khieu Awardee, Presidents Undergraduate Fellowship Award	1988
Tam Nguyen Awardee, Presidents Undergraduate Fellowship Award	1989
Dung Pham Awardee, Presidents Undergraduate Fellowship Award	1989
Diephung Nguyen Awardee, Presidents Undergraduate Fellowship Award	1991

GRADUATE RESEARCH TEACHING

A. Chair for Masters thesis

1. Alain Théon, DVM Chair	1988
2. Lydia Gan, MD Chair	1989

B. Member Thesis Committee

1. Mary Vassa	1988
2. Joanne Epping	1993 and 1994
3. Miriam Stoll	1995
4. Dana Osowiecki	1992-1993, 1997
5. Sadie Mills Undergraduate honors thesis: "College Honors"	1998

C. Oncology Research Fellows

1. Mary Estakhri, MD 1986
2. Jonathan Wardell, MD 1987
3. James C. Alex, MD 1992-1993
   a. Resident Prize for Research 1994
      New England Cancer Society
   b. First prize for research presentation 1994
      New England Otolaryngologic Society
   c. Finalist, Residents Award Competition 1994
      Triological Society.
4. Christine Gourin, MD 1992-1993
5. Michael Curran, MD1996
   a. Awardee, Roger S. Allbee Research Fellowship
6. Dr. Hella Gollasch
   a. Fellowship award (DAAD:German Academic Exchange Program for Postdoctoral Fellows) 1996-1997

D. Medical Student Research Adviser

1. Melissa Volansky 1993
2. Suzanne (Elise) Ames 1995

CONTRACTS AND GRANTS

PI: Krag
Agency: American Cancer Society
Title: "Clinical Oncology Career Development Award"
Amount: $90,000
Period: 1987-1990

PI: Krag
Agency: Cytogen
Title: "Imaging Ovarian Cancer with Radiolabeled Monoclonal Antibody"
Amount: $45,000
Period: 1989-1990

Co-PI: Krag
Agency: Cytogen
Title: "Imaging Colorectal Cancer with Radiolabeled Monoclonal Antibody"
Amount: $20,000
Period: 1989-1990

PI: Krag
Agency: Cytogen
Title: "Imaging Ovarian Cancer with Radiolabeled Monoclonal Antibody and Intraoperative Detection"
Amount: $52,000
Period: 1990-1991

PI: Krag
Agency: Cytogen
Title: IN-18: "Imaging Colorectal Cancer with Radiolabeled Monoclonal Antibody and Intraoperative Detection"
Amount: $88,000
Period: 1991-1992

PI: Krag
Agency: Cytogen
Title: 372 IN-10: "Dose Range Efficacy and Safety of Intravenously Administered 111-IN-CYT-372 in the Imaging of Colorectal Cancer" 6/92-12/93
Amount: $10,500
Period: June 1992-December 1993

PI: Krag
Agency: Zeneca
Title: Protocol 1033IL/0004
Amount: $3,050
Period: 1993

PI: Krag
Agency: National Surgical Adjuvant Breast and Bowel Project
Title: 1) Breast Cancer Prevention Trial
4 2) Breast and bowel cancer treatment protocols
Amount: $192,500
Period: (May 1992-Present)

PI: Krag
Agency: Department of Defense (DOD)
Title: Targeting Breast Cancer with Small Ligands
Grant #: DAMD17-94-J-4373
Amount: Total: $893,695 Direct: $598,217
Period: 8/31/94-8/30/98

PI: Krag
Agency: National Cancer Institute (NCI)
Title: Minimal Access Surgery for Staging of Breast Cancer
Grant #: U01CA65121
Amount: Total $1,053,148 Direct: $762,875
Period: 9/16/94-8/31/97

PI: Krag
Agency: Supplement:National Cancer Institute (NCI)
Title: Fluorescence insitu hybridization study for chromosomal aberration in primary breast tumor and lymph node metastasis
Grant #: U01CA65121
Amount: $24,629 direct
Period: 9/16/94-8/31/97

PI: Krag
Agency: Supplement:National Cancer Institute (NCI)
Title: Reverse transcriptase polymerase chain reaction (PCR) analysis of the sentinel nodes
Grant #: U01CA65121
Amount: $24,946 direct
Period: 9/16/94-8/31/97

PI: Berta Geller, PhD (Krag Co-investigator/Surgery Coordinator (2% effort)
Agency: National Cancer Institute (NCI)
Title: Vermont Breast Cancer Surveillance System
Grant #: N01CA70013
Amount: Total: $520,679 Direct$347,157
Period: 9/30/95-7/31/00 (through '98 for Krag only) - 0$ received '97 & '98)

PI: Krag
Agency: Allegheny-Singer Research Institution/NCI
Title: BCPT ancillary study to assess the effects of tamoxifen on the hemostatic system and on cholesterol levels
Grant #: DA0026
Amount: Total: $77,757 Direct: $70,688
Period : 8/15/96-5/31/97

PI: Bruce Compas, PhD (Krag Co-investigator (2.5% effort)
Agency: National Cancer Institute
Title: Comparison of Psychosocial Intervention in Breast Cancer
Grant #: R01 CA67936
Amount: Total year 1 = $369,622
year 2 = $361,599 (Total = $1,844,286)
year 3 = 360,637
year 4 = 370,917
Year 5 = 381,511
Period: 6/1/97-5/31/02

PI: Krag
Agency: National Cancer Institute(NCI)
Title: The sentinel node in breast-conserving therapy
Grant #: IU 01CA74137
Amount: Total: $2,055,741
year 1 = $647,288
year 2 = $701,213
year 3 = $707,240
Period: 7/1/98-6/30/01

PI: Krag
Agency: Integrated Therapeutics Group, Inc. (Sunbelt Melanoma)
Title: A multicenter trial of adjuvant interferon α-2B for melanoma patients with early lymph node metastases detected by lymphatic mapping and sentinel lymph node biopsy
Protocol #: 695
Amount: Total: $28,125 Direct:$21,990
Period: 3/24/98 - indefinitely

PI: Krag
Agency: DHHS PHS NIH NCI
Title: Targeting Breast Cancer with Small Ligands
Grant #: 1 R01 CA807790
Amount: Total: $898,175 Direct:: $592,923
Period 4/15/99-3/31/02

PI: Krag
Agency: US Army Medical Research and Materiel Command (USAMRMC), Congressionally-Directed Medical Research Programs' Breast Cancer Research Program (BRCP)
Title: Identification of small ligands targeting breast cancer by *in vivo* screening of peptide libraries in breast cancer patients
Grant #: BC980739
Amount: Total: $1,708,901 Direct: $1,133,073
Period: 7/1/99-6/30/02

PI: Krag
Agent: Zeneca Pharmaceuticals/AstraZeneca
Title: Cancer Research Fund
Status: Unrestricted/one-time gift
Amount: $40,000
Period: 8/31/99 - indefinite

PI: Krag
Agent: NCI
Title: Cancer cells in blood
Grant #: I U01CA74137 (Administrative supplement)
Amount: $45,000
Period: 7/1/99-6/30/00

PI: Krag
Agent: ChromaVision
Title: VCC-D Krag Research Fund
Status: One-time gift
Amount: $60,000
Period: 4/00 - indefinite

Grants Submitted and Pending:

PI: Krag
Agency: NCI
Title: Identification of small peptides which target cancer cells isolated from blood of patients
Grant #: 1030179440
Amount: Total: 226,500 Direct: $150,000
Period: 7/01/00-6/30/02

NATIONAL CLINICAL TRIALS ACTIVITIES:

Principal investigator at University of Vermont for all NSABP National Surgical Adjuvant and Breast and Bowel Project clinical trials. 1992-April 1997

Principal Investigator at University of Vermont for all CALGB Cancer and Leukemia Group B clinical trials. 1994-1996

Protocol Chairman- Multicenter clinical trial of Minimal Access Surgical Staging of Breast Cancer 1994-1998

Protocol Chairman- Multicenter clinical trial of Minimal Access Surgical Staging of Malignant Melanoma 1994-Present

Protocol Chairman- 1998-Present
A Randomized Phase III Clinical Trial to Evaluate Sentinel Node Resection Compared to Conventional Axillary Lymphadenectomy in Clinically Node-Negative Breast Cancer Patients (Joint study through University of Vermont and NSABP sponsored by NCI)

BIBLIOGRAPHY FOR DAVID N. KRAG, MD, FACS

A. PUBLISHED WORK

1981

1. Keefer J , Barish K, Krag DN and Kay J. Alveolar-arterial 02 difference: A reliable index of Qs/Qt? Proceedings of the American Association of Anesthesia, p27. ABSTRACT

1982

2. Krag DN and Stansel H. Popliteal synovial cyst causing complete occlusion of the popliteal artery. J Bone Joint Surg. RESEARCH ARTICLE

1984

3. Krag DN, Storm FK, Kern DH, and Morton DL. Indomethacin blocks thermotolerance in human malignant cells. Proceedings of the 32nd Meeting of the Radiation Research Society. p52. ABSTRACT

4. Storm FK, Krag DN, Kaiser LR, Silberman AW, and Morton DL. Localized hyperthermia in phantoms, animals and human clinical trials. Proceedings of the 20th Annual Meeting of the Amer Soc Clin Oncol. p259. ABSTRACT

5. Krag DN, Storm FK, Kem DH, GL Kaufman and Morton DL. Natural thermoresistance and acquired thermotolerance: Etiology and prevention in vitro. Proceedings of the 4th International Symposium on Hyperthermic Oncology, Aarhus, Denmark. July 1984. #96. ABSTRACT

6. Snow DH, Krag DN, Silberman AW and Storm FK. Hyperthermia and reduction of blood supply for treatment of cancer. Pulse 26:12. RESEARCH ARTICLE

1985

7. Chase D, Krag DN, Kem DH, Storm F and Morton DL. Video time-lapse monitoring of acute effects of hyperthermia on cells in culture. Proceedings of the 5th Annual Meeting of the North American Hyperthermia Group, p3. ABSTRACT

8. Silberman AW, RW Rank, Krag DN, Storm FK, Benz M, Drury B and Morton DL. Effect of localized magnetic induction hyperthermia on the brain: Temperature versus intracranial pressure. Proceedings of the 33rd Meeting of the Radiation Research Society, p25. ABSTRACT

9. Krag DN, Storm FK, Kem DH, and Morton DL. Temperature modulation of PGE2 release by human malignant cells in vitro. Proceedings of the 5th Annual Meeting of the North American Hyperthermia Group, p5. ABSTRACT

10. Krag DN, Chase D, Goffney W, Kem DH, Storm FK and Morton DL. Short-term rhodamine 123 (R 123) exposure acts as a potent thermic sensitizer. Proceedings of the 76th Annual Meeting of the American Association of Cancer Research. May 1985, p239; #941. ABSTRACT

1986

11. Silberman AW, RW R and, Krag DN, Storm FK, Benz M, Drury B and Morton DL. Effect of localized magnetic-induction hyperthermia on the brain. Cancer 57:1401-1404. RESEARCH ARTICLE

12. Goffney WH, Kem DH, Chase D, Krag DN, and Storm FK. Rhodamine 123 combined with hyperthermia retards the growth of a human melanoma implanted in nude mice. American College of Surgeons, 71st Annual Clinical Congress, Surgical Forum, Volume XXXVII, October. p421. RESEARCH ARTICLE

13. Krag DN, Storm FK, Drury B, Benz M, J Krag and Morton DL. Hyperglycemic hyperthermia: Using the glucose clamp technique (GCT). Proceedings of the 34th Annual Radiation Research Society, Philadelphia PA. April 1986. p14; Bb 10. ABSTRACT

1987

14. Estakhri M, Ulmer J, Théon A, Chase D, Twomey P and Krag DN. Selective retention of rhodamine 123 by spontaneous tumors in outbred animals. 1987 Proceedings of the 78th Annual Meeting of the American Association for Cancer Research, p437, #6788. ABSTRACT

15. Krag DN and Chase D. Survival of human malignant melanoma cells exposed to rhodamine 123 for 24 hours is markedly decreased by small increases in temperature. 1987 Proceedings of the 78th Annual Meeting of the American Association for Cancer Research, p432; #1713. ABSTRACT

16. Lameh J, Chuang RY, Krag DN and Chase D. The Inhibitory effect of Rhodamine 123 on DNA and RNA synthesis of E.coli in vitro. Proceedings of the Western Pharmacology Society; 30:103-107. ABSTRACT

17. Chase D, Krag DN, Johnson L and Storm FK. Rhodamine 123 significantly alters mitochondrial morphology and viability in human melanoma cells but not in Chinese hamster ovary cells. Proceedings of the 78th Annual Meeting of the American Association for Cancer Research, p432; #1714. ABSTRACT

18. Chuang RY, Krag DN, Chase D and Chuang LF. Rhodamine 123 as a cancer chemotherapeutic agent. Studies on its interaction with leukemia DNA and RNA polymerases. Proceedings of the 78th Annual Meeting of the American Association for Cancer Research, p308; #1219. ABSTRACT

1988

19. Kern DH, Krag DN, Kaufman GL, Morton DL and Storm FK. Thermoresistance of human malignant melanoma modulated prostaglandin E2. J Surg Oncol 37:60-64, January. RESEARCH ARTICLE

20. Théon A and Krag DN. Effects of tumor necrosis factor and hyperthermia on B-16 mouse melanoma cells in vitro. Proceedings of the 8th Annual Meeting of the North America Hyperthermia Group, Houston, TX. p40. ABSTRACT

21. Krag DN, Gan L, and Théon A. Enhancement of rhodamine 123 cytotoxicity by hyperthermia. Proceedings of the 8th Annual Meeting of the North America Hyperthermia Group p37. ABSTRACT

22. Woo SY, Van Kersen I, Anderson RL, Krag DN, Kapp DS, Rice GC, and Hahn GM. Heterogeneity of heat responses in tumors. Proceedings of the 8th Annual Meeting of the North America Hyperthermia Group p12. ABSTRACT

23. Estakhri M, Blumenthal S, Twomey P, Krag DN and Chase D. Therapeutic gain in vitro by heat and Rhodamine 123. Proceedings of the 8th Annual Meeting of the North America Hyperthermia Group p8. ABSTRACT

24. Wardell JW, Gan L, Théon A, and Krag DN. Verapamil reversal of Rhodamine 123 resistance in B16F10 mouse melanoma cells. Proceedings of the 79th American Association of Cancer Researchers. p475 . ABSTRACT

25. Krag DN, Théon A, Gan L and Tao Z. Relationship between cellular accumulation of Rhodamine 123 and cytotoxicity in B16 melanoma cells. 22nd Annual Meeting of the Association for Academic Surgery, p100. ABSTRACT

26. Théon A, Madewell BR, Moore AS, Stephens CH and Krag DN. A pilot study of hyperthermia and intratumoral cisplatin chemotherapy in spontaneous pet animal tumors. Proceedings of the 8th Annual Conference, Veterinary Cancer Society. ABSTRACT

1989

27. Edwards B, Gan L, Twomey P and Krag DN. Quinidine enhances Rhodamine 123-mediated phototoxicity in a new Rhodamine-resistant cell line. Joint meeting of American Society for Cell Biology w. American Society for Biochemistry & Molecular Biology. 1989, p417a; #2380. ABSTRACT

28. Krag DN, A Théon, Gan L, Wardell J and Zhen S. Relationship between cellular accumulation of rhodamine 123 and cytotoxicity in B16 melanoma cells. J Surg Res 46:361-365.
RESEARCH ARTICLE

1990

29. Goffney WH, Wong J, Kem D, Chase D, Krag DN, and Storm FK. In vitro and in vivo cytotoxicity of rhodamine 123 combined with hyperthermia. Cancer Res 50:459-463.
RESEARCH ARTICLE

30. Krag DN, Théon AP, Schneider PD and Goodnight JE. Intralesional cis-diamminedichloroplatinum and purified collagen treatment of human metastatic malignancies: A feasibility study, J Surg Oncol 43:83-87.
RESEARCH ARTICLE

31. Krag DN, Théon A, and Gan L. Hyperthermic enhancement of rhodamine 123 cytotoxicity in B16 mouse melanoma cells *in vitro*. Cancer Res, 50:2385-2389. RESEARCH ARTICLE

32. Krag DN, Storm FK and Morton DL. Induction of transient hyperglycemia in cancer patients. Int J Hyperthermia. Vol. 6 (4):741-744. RESEARCH ARTICLE

33. Krag DN, Théon AP, Gan L, Wardell J, Zhen S. Reversal of thermochemotherapeutic drug resistance with verapamil. Int J Hyperthermia 6 (5):933-941.
RESEARCH ARTICLE

34. Gan L, Krag DN, Théon AP, Zhen TS and Wardell J. Reversal of thermochemotherapeutic drug resistance by Verapamil. Poster presentation at 10th Annual Meeting North American Hyperthermia Group, April 1990. p024 ABSTRACT

35. Leach MW, Higgins RJ, Boggan JE, Elkhaled S, Smith KM, Krag DN and Lee S, Theilen GH. Effectiveness of a new chlorine compound in photo dynamic therapy of the subcutaneous 9L rat glioma. 3rd Biennial Meeting of the International Photodynamic Association. p37; #32.
ABSTRACT

36. Mann WJ, Surwit EA, Krag DN, Katterhagen JG, Gallion H and Delgado G. immunoscintigraphy of ovarian cancer with indium IN-111-CYT-103. Biology and Therapy of Ovarian Cancer Meeting sponsored by Fox Chase Cancer Center, Marble Island Resort, October, 1990.
ABSTRACT

37. Théon AP, Pascoe JR, Krag DN. Intraoperative cisplatin chemotherapy for the treatment of equine malignancies. Proceedings for the 10th Annual Conference, Veterinary Cancer Society, Auburn, AL. 1990. p131 ABSTRACT

38. Théon AP, Madewell BR, Kraegel S, Marks S, Peaston A, Krag DN. Irradiation and intratumoral cisplatin chemotherapy: A pilot study in spontaneous canine tumors. Proceedings of the 10th Annual Conference, Veterinary Cancer Society, Auburn AL. 1990. p131. ABSTRACT

1991

39. Woo SY, Anderson RL , Kapp DS, Van Kerson I, Rice GC, DN Krag, Hahn GM. Heterogeneity of heat response in murine canine and human tumors: Influence on predictive assays. Int J Radiat Oncol, Biol, Phys 20:479-488. RESEARCH ARTICLE

40. Krag DN, Klein H, Schneider PD, Goodnight JE. The Role of composite tissue transfer (CTT) in limb salvage surgery. Arch Surg 126:639-641. RESEARCH ARTICLE

41. Doerr RJ, Abdel-Nabi H, Krag DN, Mitchell E. Radiolabeled antibody imaging in the management of colorectal cancer: Results of a multicenter clinical study. Ann Surg 214, 2:118-124. RESEARCH ARTICLE

42. Théon AP, Madewell BR, Moore AS, Stephens C, Krag DN. Localized thermocisplatin therapy: A pilot study in spontaneous canine and feline tumors. Int J Hyperther. Vol.7, 6:881-892. RESEARCH ARTICLE

43. Wolf RF, Goodnight JE, Krag DN, Schneider PD. Non-colorectal liver metastases: results of resection and proposed guidelines for patient selection. Surgery, Gynecology & Obstetrics 173:454-460. RESEARCH ARTICLE

44. Krag DN, Haseman MK, Ford P, Smith L, Taylor MH, Schneider PD. Intraoperative detection of ovarian and colorectal cancer using a hand-held gamma counter in diagnostic uses of radiolabeled antibodies. Haseman MK (ed), Frontiers in Nuclear Medicine p163-170. RESEARCH ARTICLE

45. Krag DN, Ford P, Smith L, Taylor M, Schneider P, Goodnight JE. Clinical immunoscintigraphy of recurrent ovarian cancer with 111IN-CYT-103. 6th International Monoclonal Antibody Immunoconjugates for Cancer, San Diego, California. ABSTRACT

1992

46. Krag DN. Antibody imaging for ovarian cancer. Applied Radiology 21, No 3: p30-33, March. RESEARCH ARTICLE

47. Collier BD, Abdel-Nabi H, Doerr RJ, Harwood JO, Kaplan EH, Winzelberg GG, Grossman SJ, Krag DN, Mitchell EP Immunoscintigraphy with 111 In-Cyt-103 in the management of colorectal carcinoma: A comparison with computed tomograph. Radiology 185:179-86. RESEARCH ARTICLE

48. Krag DN, Ford PV, Patel M, Schneider PD, Goodnight JE. Gamma probe-guided bone biopsies: A simplified technique to biopsy abnormal bony radiolocalizations. Surg Oncol 1 (5):371-377. RESEARCH ARTICLE

49. Krag DN, Haseman MK, Ford P, Smith L, Taylor MH, Schneider P, Goodnight JE. Gamma-probe localization of 111indium-labeled B72.3: An extension of immunoscintigraphy. J of Surg Oncol 51:226-230. RESEARCH ARTICLE

1993

50. Théon AP, Pascoe JR, Carlson GP, Krag DN. Intratumoral chemotherapy with cisplatin in oily emulsion for treatment of tumors in horses. Journal of the American Veterinary Medical Association: 202 (2):261-267. RESEARCH ARTICLE

51. Zulim RA, Rocco M, Goodnight Jr. JE, Smith GJ, Krag DN, and Schneider PD. Intraoperative autotransfusion in hepatic resection for malignancy: Is it safe? Arch Surg 128:206-211. RESEARCH ARTICLE

52. Krag DN. Clinical utility of immunoscintigraphy in managing ovarian cancer. J Nucl Med 34:545-548. RESEARCH ARTICLE

53. Krag DN, Ford P, Smith L, Taylor M, Schneider PD, Bushberg JT, Goodnight JE. Clinical immunoscintigraphy of recurrent ovarian cancer with 111indium-labeled B72.3 monoclonal antibody. Arch Surg 128:819-823. RESEARCH ARTICLE

54. Alex JC, Krag DN. Gamma probe-guided localization of lymph nodes. Surg Oncol 2:137-143. RESEARCH ARTICLE

55. Alex JC, Weaver DL, Fairbank JT, Krag DN. Gamma-probe-guided lymph node localization in malignant melanoma. Surg Oncol 2:303-308. RESEARCH ARTICLE

56. Krag DN, Weaver DL, Alex JC, Fairbank JT. Surgical resection and radiolocalization of the sentinel lymph node in breast cancer using a gamma probe. Surg Oncol 2:335-340. RESEARCH ARTICLE

57. Krag DN. Cancer prevention & control: Breast care and treatment. Perspectives. Vol 4, No 4; Vermont Department of Health. INVITED COMMENTARY

58. Krag DN, DL Morton, and FK Storm. Magnetic induction heating of large visceral tumors. Hyperthermia: Past experience and future prospects, George M. Hahn Symposium, Palo Alto, California. p18 (S-7). ABSTRACT

59. Gourin CG, Gan L, Vichi P, Tritton T, Krag DN. Efflux of fura-2 from multidrug-resistant B16 cells: reversal by verapamil and organic anion transport inhibitors. 1996 Spring Meeting - New England Cancer Society, Boston, MA. p33 ABSTRACT

60. Alex JC, Weaver DL, Fairbank JT, Krag DN, Rankin B. Gamma-probe-guided localization of lymph nodes in malignant melanoma. 104th Annual Fall Meeting - New England Cancer Society, Boston, MA. p27 ABSTRACT

61. Surwit EA, Childers JM, DN Krag, Katterhagen JG, Gallion HH, Waggoner S, Mann WJ. Clinical assessment of 111IN-CYT-103 immunoscintigraphy in ovarian cancer. Surgery, Gynecology and Obstetrics 48:285-292. RESEARCH ARTICLE

1994

62. Gourin CG, Gan L, Vichi P, Tritton TR, Krag DN. Efflux of fura-2 from multidrug resistant B16 cells: reversal by verapamil and organic anion transport inhibitor. Cellular Pharmacology 2:115-120. RESEARCH ARTICLE

63. Oligino L and Krag, DN. Targeting breast cancer with small ligands. 1994 Regional Cancer Research Symposium, Vermont Cancer Center, Burlington, VT. October. #33. ABSTRACT

64. DN Krag, DL Weaver, JC Alex, JT Fairbank. Gamma probe-guided radiolocalization of the sentinel lymph node. VI-Annual Symposium[of Diagnostic and Therapeutic Application of Radiolabeled Antibodies and Receptors for Diagnostic and Therapeutic Purposes, University of Miami School of Medicine, Key Biscayne, FL. p94. ABSTRACT

65. Krag DN, Weaver DL, Alex JC, Fairbank JT. Surgical resection and radiolocalization of sentinel lymph node in breast cancer using a gamma probe. Oral presentation at 47th Annual Meeting of the Society of Surgical Oncology, Houston, TX. 1994. p32. ABSTRACT

66. Krag DN, Alex JC. Gamma probe-guided localization of the sentinel lymph node in malignant melanoma patients. CME/Gamma Probes of Intraoperative Detection of Radiolabeled Tissues, University of Vermont. March. ABSTRACT

67. Alex JC, Krag DN. Minimizing iatrogenic trauma during surgery for melanoma of the head and neck. The 2nd John H. Davis Society Scientific Symposium, Stowe, VT. June. ABSTRACT

68. Alex JC, Sofferman RA, Millay DJ, Rawlings MS, Krag DN. Sentinel node localization in head and neck melanoma. Eastern Section, the American Laryngological, Rhinological, and Otological Society. ABSTRACT

1995

69. Krag DN, Meijer SJ, Weaver DL, Loggie BW, Harlow SP, Tanabe KK, Laughlin EH, Alex JC. Minimal access surgery for staging malignant melanoma. Arch Surg 130:654-661. RESEARCH ARTICLE

70. Krag DN, Meijer S, Weaver D, Loggie B, Laughlin E, Alex J. Minimal access surgery for staging regional lymph nodes in malignant melanoma. Society of Surgical Oncology 48th Annual Cancer Symposium, Boston, MA. March 1995. p29. ABSTRACT

71. Moffat Jr. FL, Franceschi D, Krag DN, Robinson DS, Temple WJ. Symposium: In situ and microinvasive carcinoma of the breast. Contemporary Surgery 7(2):109-115. INVITED COMMENTARY

72. Krag DN: Sentinel node dissection appears safe and effective in preventing melanoma metastases. Skin and Allergy News. March issue. MEDICAL NEWSPAPER

73. Krag DN: Ultrasound margins in breast surgery. EARL:Administrative Radiology Journal September; XIV (I):33-34. INVITED ARTICLE

74. Gourin CG, Krag DN. Targeted imaging of colorectal cancer. Vladimir P Torchilin, PhD, (ed): Handbook of Targeted Delivery of Imaging Agents. CRC Press, Boca Raton FL. pp229-249. BOOK CHAPTER

75. Krag DN and Schlegel ME. Antibody-mediated imaging of ovarian cancer. Vladimir P Torchilin, PhD (ed): Handbook of Targeted Delivery of Imaging Agents. CRC Press, Boca Raton FL. pp177-192. BOOK CHAPTER

76. Krag DN. Minimal access surgical staging of breast and melanoma. General Surgery and Laparoscopy News. May, 1995 issue. MEDICAL NEWSPAPER

77. Krag DN. Radiolabeled Lymph Node Tracing. EARL:Administrative Radiology Journal November; XIV (XI):62-63. INVITED ARTICLE

78. Sofferman RA, Johnson DL, and Krag DN. Laryngeal mask airway. Otolaryngology-Head and Neck Surgery 113:502-507. RESEARCH ARTICLE

1996

79. Krag DN, Alex JC. The gamma-probe technology for defining lymphatic anatomy and the sentinel nodes. Surg Oncol Clinics of North America: Lymph nodes in Surg Oncol. Roger S. Foster (ed). W.B. Saunders, Philadelphia PA; January; Vol 5; pp33-41. BOOK CHAPTER

80. Dowlat K, Fan M, Rayudu G, Krag D, Shekarloo M, and Bloom K. Diagnostic value of axillary sentinel node in operative breast cancer. The Breast Journal. January/February issue. p26 ABSTRACT

81. Sofferman RA, Nathan MH, Fairbank JT, Foster, Jr RS, Krag DN. Preoperative Technetium-99m-sestamibi imaging: Paving the way to minimal access parathyroid surgery. Archives of Otolaryngology 122:369-374. RESEARCH ARTICLE

82. Krag D, Harlow S, Weaver D. Primary node biopsy and breast cancer. American College of Surgeons Clinical Congress, October 1996, San Francisco CA. Postgraduate Course Manual, "Breast Disease." LIMITED DISTRIBUTION

1997

83. Crews KA, Kuhn JA, Fisher TL, Grant MD, Peters GN, Knox SM, Krag DN. Sentinel node biopsy for breast cancer:Preliminary results. 36th Annual Northern Texas Chapter American College of Surgeons Meeting, February 1997, Dallas, TX. ABSTRACT

84. Shriver C, Balingit AG, Caravalho J, Rodriguez AA, Jacques DP, Maniscalco-Theberge M, Deavers M, and Krag DN. Unfiltered Tc-99m-sulfur colloid lymphoscintigraphy and gamma-probe in detection of sentinel node in breast cancer patients. Society of Nuclear Medicine 44th Annual Meeting San Antonio TX. June. #15862. ABSTRACT

85. Alex J, Krag D, Harlow S, Meijer S, Loggie B, Kuhn J, Gadd M. Gamma probe radiolocalization of regional lymph nodes in head & neck melanoma. American Academy of Facial, Plastic & Reconstructive Surgery. September. #670. ABSTRACT

86. Gulec SA, Moffat FL, Carroll RG, Krag DN. Gamma probe-guided sentinel node biopsy in breast cancer. The Quarterly Journal of Nuclear Medicine 41:251-61. RESEARCH ARTICLE

87. Miner T, Midkiff R, Stoldt K, Jacques D, Krag D, Shriver C. The use of ultrasound and gamma camera imaging in primary lymph node resection for breast cancer. The 50th Annual Meeting of the Society of Surgical Oncology , Chicago, IL, March. p38. ABSTRACT

88. Gulec SA, Moffat FL, Sarafini AN, Sfakianakis GN, Boggs J, Allen L, Escobedo D, Sundaram M, Boyle M, Livingstone A, Krag D. Sentinel node localization in patients with breast cancer. Society of Nuclear Medicine 44th Annual Meeting , San Antonio, TX. June. #69354. ABSTRACT

89. Moffat FL, Gulec SA, Sfakianakis GN, Sittler SY, Allen L, Serafini AN, Pruett CS, Robinson DS, Livingstone AS, Krag DN. Sentinel node biopsy for breast cancer using unfiltered 99mTc sulfur-colloid and gamma probe. Presentation 47th Society of Pelvic Surgeons Annual Meeting, Indianapolis IN, November. ABSTRACT

90. Krag DN, Harlow SP, Weaver DL, and Ashikaga T. Technique of selected resection of radiolabeled lymph nodes in breast cancer. 20th Annual San Antonio Breast Cancer Symposium. December. p40. ABSTRACT

91. Oligino L, Sastry L, Lung T, Bigelow J, Cao T, Burke T, Wang S, Krag D, Roller P and King CR. Identification of a non-phosphorylated small peptide ligand of the Grb2 SH2 domain. J Biological Chemistry. 272(46):29046-52, 1997. RESEARCH ARTICLE

1998

92. Krag DN. Gamma probe-guided resection of the radiolabeled sentinel lymph node in breast cancer. Breast Disease: A Year Book Quarterly 8(4):300-301. INVITED EDITORIAL

93. Crews KA, Kuhn JA, Fisher TL, Grant MD, Peters GN, Knox SM, Krag DN. Sentinel node biopsy for breast cancer:Preliminary results. 66th Southeastern Congress, February 1998, Atlanta, GA ABSTRACT

94. Smith PAF, Krag DN and Weaver DL. Decreased histologic accuracy of detecting axillary node metastases in breast cancer patients may follow submission of nodal tissue for ancillary studies. 87th Annual Meeting of the United States and Canadian Academy of Pathology, March 1998, Boston, MA. Mod Pathol 11(1): 28A. ABSTRACT

95. Pijpers R, Krag D, Borgstein P, Greuter H, Teule G, Meijer S. Influence of the applied tracer on nodal uptake in sentinel node biopsy in melanoma. European Association of Nuclear Medicine Congress, Glasgow, United Kingdom. Eur J Nucl Med 24:992. August. ABSTRACT

96. Miner T, Flicek P, Jacques D, Krag D, Shriver C. Guidelines for the safe management of tissues from radiolocalized sentinel lymph node biopsies. 5lst Annual Meeting of the Society of Surgical Oncology, Chicago IL, March. p13. ABSTRACT

97. Alex JC, Krag DN, Harlow SP, Meijer SJ, Loggie BW, Kuhn J, Gadd M. Localization of regional lymph nodes in melanomas of the head and neck. Archives of Otolaryngology:Head and Neck. February 124:135-140. RESEARCH ARTICLE

98. Krag DN, Harlow SP, Weaver DL, Ashikaga T. Technique of selected resection of radiolabeled lymph nodes in breast cancer patients. Seminars in Breast Disease:Radiologic, Pathologic, and Surgical Considerations. June 1(2):1-6 INVITED RESEARCH ARTICLE

99. Rubio IT, Korourian S, Cowan C, Krag DN, Colvert M, Klimberg VS. Sentinel lymph node biopsy for staging breast cancer. Amer J Surg 176:532-537. RESEARCH ARTICLE

100. Krag DN, Ashikaga T, Harlow SP, Weaver DL. Development of sentinel node targeting technique in breast cancer patients. The Breast Journal. 42(March-April):67-74.
RESEARCH ARTICLE

101. Ames SE, Krag DN, and Brady MS. Radiolocalization of the sentinel lymph node in Merkel cell carcinoma: A clinical analysis of seven cases. J Surg Oncol 67 (April):251-254.
RESEARCH ARTICLE

102. David Krag, Seth Harlow, Donald Weaver and Taka Ashikaga. Technique of sentinel node resection in melanoma and breast cancer:Probe-guided surgery and lymphatic mapping. Eur J Surg Oncol 24(2):89-93. INVITED RESEARCH ARTICLE

103. Gulec S, Moffat F, Serafini A, Sfakianakis G, Allen L, Boggs J, Escobedo D, Livingstone A, Carroll R, Krag D. Sentinel Lymph node localization in early breast cancer. J Nucl Med 39:1388- 1393.
RESEARCH ARTICLE

104. Pijpers R, Borgstein PJ, Meijer S, Krag DN, Hoekstra OS, van Diest P, Greuter H, and Teule GJJ. Transport and retention of colloidal tracers in regional lymphoscintigraphy: Influence on lymphatic mapping and sentinel node biopsy. Melanoma Res 8:413-418. RESEARCH ARTICLE

105. Krag DN et al (multi authors). The sentinel node in breast cancer: A Multicenter Validation Study. N Engl J Med 339(14):941-946 RESEARCH ARTICLE

106. Krag DN. Minimal access surgery for staging regional lymph nodes: The sentinel-node concept. Current Problems in Surgery 35 (Nov.):951-1018. INVITED RESEARCH ARTICLE

107. Rubio IT, Korourian S, Cowan C, Krag DN, Colvert M, Klimberg VS. Use of touch preps for intraoperative diagnosis of sentinel lymph node metastases in breast cancer. The American Journal of Surgery 5(8):689-694. RESEARCH ARTICLE

108. Miner T, Shriver C, Jaques D, Maniscalco-Theberg M, and Krag D. Ultrasonographically guided injection improves localization of the radiolabeled sentinel lymph node in breast cancer. Ann Surg Oncol 5(4):315-321. RESEARCH ARTICLE

1999

109. Gogel BM, Kuhn JA, Ferry KM, Fisher TL, Preskitt JT, O'Brien JC, Zelig HL, Stephens JS, Krag DN. Sentinel lymph node biopsy for melanoma. Amer J Surg 176(6):544-547, 1998.
RESEARCH ARTICLE

110. Krag DN. Commentary for Oncology on Sentinel Lymph Node Mapping in Breast Cancer; Cody III HS, author. Oncology 13(1):35-36. INVITED COMMENTARY

111. Weaver DL, Krag DN, Ashikaga T, Harlow SP: Occult metastases in breast cancer are more likely to be detected in sentinel lymph nodes than in non-sentinel lymph nodes. Oral presentation at United States and Canadian Academy of Pathology 88th, San Francisco CA Annual Meeting, March 22, 1999. Mod Pathol 12(1):33A. ABSTRACT

112. Krag DN, Moffat, Jr. FL, Ashikaga T. Letter to the Editor: Reply. New Engl J Med 340:317-319. INVITED EDITORIAL

113. Miner TJ, Shriver CD, Flicek PR, Miner FC, Jaques DP, Maniscalco-Theberge ME, Krag DN. Guidelines for the safe use of radioactive materials during the localization and resection of the sentinel lymph node. Annals of Surg Oncol 6(1):75-82. RESEARCH ARTICLE

114. Krag DN. Editorial for Veronesi article: Current status of sentinel lymph node surgery for breast cancer. J Na Cancer Inst 91(4; Feb. 17):302. INVITED EDITORIAL

115. Cushman M, Costantino JF, Bovill EG, et al (Krag DN),. Tamoxifen and inflammation. ASCO annual meeting, May 1999. ABSTRACT

116. Feldman S, Krag DN, McNally R, Moor B, Klein P. Limitation in gamma probe localization of the sentinel node in breast cancer patients with large excisional biopsy. American College of Surgeons 188:248-54. RESEARCH ARTICLE

117. Gulec S, et al (DN Krag). Sentinel lymph node localization in early breast cancer. J Nucl Med. Letter to the Editor #R8-644. January 1999. LETTER TO EDITOR

118. Primo K, Compas BE, Oppedisano G, Howell DC, Epping-Jordan JE and Krag DN. Intrusive thoughts and avoidance in breast cancer:individual differences and association with psychological distress. Psychology and Health 15:1-13. RESEARCH ARTICLE

119. Oligino L, Kingsley SL, Gollasch H, King CR, Roller P, Krag D. Targeting breast cancer with small ligands. Vermont Cancer Center, Regional Cancer Research Symposium, Burlington, VT. Poster Abstract #13. ABSTRACT

120. Harlow S, Krag D, Weaver D and Ashikaga T. Extra-axillary sentinel lymph nodes in breast cancer. Breast Cancer/Japan 6(2):159-65. RESEARCH ARTICLE

121. Cushman M, Costantino JP, Bovill EG, Tracy RP, Wickerham DL, Roberts JD, Buckley L, Krag DN. Effect of tamoxifen on cardiac risk factors in women without cancer: the Breast Cancer Prevention Trial. Proc ASCO 18:75a. #283. RESEARCH ARTICLE

122. Krag DN, Beitsch PD, Moss TJ, et al. Breast cancer cells in the blood. 22nd Annual San Antonio Breast Cancer Symposium, San Antonio, TX. Poster abstract #505. December 1999. ABSTRACT

123. Krag DN. The sentinel node for staging breast cancer:current review. Breast Cancer/Japan 6:233-236. RESEARCH ARTICLE

124. Smith PAF, Harlow SP, Krag DN and Weaver DL. Lymph node tissue for ancillary studies decreases accuracy of conventional breast cancer axillary node staging. Mod Pathol 12(8):781-85.
RESEARCH ARTICLE

125. Harlow SP, Krag DN , Ames SE, Weaver DL. Intraoperative ultrasound localization to guide surgical excision of impalpable breast carcinoma. J Amer Coll Surg 189:241-46.
RESEARCH ARTICLE

126. Krag DN and Moffat FL, Jr. Nuclear medicine and the surgeon. The Lancet 354(Sept. 18):1019-22.
RESEARCH ARTICLE

127. Cushman M, et al. (Krag DN). Effect of tamoxifen on venous thrombosis risk factors in women without cancer: The Breast Cancer Prevention Trial (BCPT). 1999 Annual Meeting of the American Society of Hematology. Abstract #4542.
ABSTRACT

128. Krag DN, Ashikaga T, Moss TJ, et al. Breast cancer cells in the blood. The Breast Journal 5(6):354-358.
RESEARCH ARTICLE

Albert GD, Alex JC, Krag DN, Weinberg DA. Gamma probe localization of cranial bone lesions. Ophthal Plast Reconstru Surg 15(6):470-472.
RESEARCH ARTICLE

130. Miner TJ, Shriver DC, Jaques DP, Maniscalco-Theberge ME, Krag DN. Sentinel lymph node biopsy for breast cancer: the role of previous biopsy on patient eligibility. AM Surg 65(6):493-8.
RESEARCH ARTICLE

131. Moffat Jr. FL, Gulec SA, Sittler SU, Sarafini AN, Sfakianakis GN, Boggs J, Franceschi D, Pruett CS, Pop R, Gurkok C, Livingstone AS, KRAG DN. Unfiltered sulfur colloid and sentinel node biopsy for breast cancer technical and kinetic considerations. Ann Surg Oncol 6(8):746-55.
RESEARCH ARTICLE

132. Epping-Jordan JE, Compas BE, Osowiecki DM, Oppedisano G, Gerhardt C, Primo K, Krag DN. Psychological adjustment to breast cancer: process of emotional distress. Health Psychology 18(4):315-26.
RESEARCH ARTICLE

133. Compas BE, Stoll MF, Thomsen AH, Oppedisano G, Epping-Jordan JE, Krag DN. Adjustment to breast cancer:age-related differences in coping and emotional distress. Breast Cancer Res Treat 54(3):195-203.
RESEARCH ARTICLE

133. Moes GS, Guibord RS, Weaver DL, Krag DN, Harlow SP. Intraoperative cytologic evaluation of sentinel lymph nodes in breast cancer patients. United States and Canadian Academy of Pathology 89th Annual Meeting, New Orleans LA. Mod Pathol 2000 13(1):28A. ABSTRACT

2000

135. Weaver DL, Krag DN, Ashikaga T, Harlow SP, O'Connell M. Pathologic analysis of sentinel and non-sentinel lymph nodes in breast cancer: a multi-center study. Cancer 88(5):1099-1107.
RESEARCH ARTICLE

136. Krag DN. Sentinel biopsy for detection of metastases. Cancer J (Boston MA)6(suppl 2):S121-124. RESEARCH ARTICLE

B. ACCEPTED FOR PUBLICATION/IN PRESS

1. Krag DN. Gamma probe-guided sentinel lymphadenectomy. International Consensus Symposium: Sentinel Lymphonodectomy in Cutaneous Malignancies, Dermatology and Allergy Clinic, Augsburg, Germany, March. Limited distribution. SUMMARY MANUSCRIPT

2. Moffat, Jr FL and Krag DN. Breast Cancer Therapy:Application of Evidence to Patient Management:Should surgeons abandon routine axillary dissection for sentinel node biopsy in early breast cancer? BOOK CHAPTER

3. KRAG DN, Harlow SP, Weaver DL, Ashikaga T. Radiolabeled sentinel node biopsy:a collaborative trial with the National Cancer Institute. World J Surg. RESEARCH ARTICLE

4. Cochran AJ, Balda B-R, Starz H, et al(Krag DN). The Augsburg consensus: a commentary on the techniques of lymphatic mapping, sentential lymphadenectomy and completion lymphadenectomy in cutaneous malignancies. Cancer. RESEARCH ARTICLE

C. SUBMITTED WORK

McMasters K, Reintgen D, Ross M, Wong S, Gershenwald J, Krag D, Noes R, Viar V, Cerrito P and Edwards M. Sentinel lymph node biopsy for melanoma: how many radioactive nodes should be removed (Pertains to Sunbelt Melanoma Trial.)? March 2000.

Cushman M. Costantino J, Tracy R, Kyunghee Song, Buckley L, Roberts J, Krag D. Tamoxifen and novel cardiac risk factors in healthy women. Annals of Internal Medicine. March 2000.

Wellman PS, Dalton EP, Krag D, Kern KA, Howe RD. Tactile imaging of breast masses: first clinical report. Archives of Surgery. April 2000.

McMasters K, Reintgen D, Ross M, Wong S, Gershenwald J, Krag D, et al. Sentinel lymph node biopsy for melanoma: how many radioactive nodes should be removed? Journal (?) March 2000.

Krag DN. Sentinel lymph node biopsy for the detection of metastases. National Oncology Forum:The Cancer Journal from Scientific American. May 2000.

LECTURES/PRESENTATIONS

1984

"Thermotolerance"
Fifth Annual Meeting of the International Clinical Hyperthermia Society,
Como, Italy.

"Magnetrode Regional Hyperthermia Multi-Institutional National Cooperative Study of 1,170 patients"
Fifth Annual Meeting of the International Clinical Hyperthermia Society,
Como, Italy.

"Hyperthermia in Cancer Therapy"
Dameron Hospital Association, Stockton, CA.

"Hyperthermia in the Treatment of Cancer"
Seminar to the Department of Anatomy and Cell Biology University of Southern CA.
"Hyperthermic Treatment of Cancer"
Seminar to the American College of General Practitioners.
1985
"Treatment Perspectives - GI Cancer"
American Cancer Society and Sacramento Oncology Nursing Society CME Seminar: A Practical Look at GI/GU Cancer.
1986
"Hyperthermic Treatment of Cancer"
University of California, Davis Medical Center, Surgical Grand Rounds.
"HG HT: Using the Glucose Clamp Technique"
North American Hyperthermia Group Annual Meeting.
1987
"Role of Hyperthermia in Cancer Treatment"
University of California, Davis, 9th Annual Surgical Postgraduate Course.
"Hyperthermia: Its Use as an Anticancer Agent"
Veterans Administration Hospital, Martinez, CA Grand Rounds.
"Hyperthermia: Its Role as a Possible Anticancer Agent"
Biomedical Engineering Graduate Group, University of California, Davis.
"The Inhibitory Effects of Rhodamine 123 on DNA and R
A Synthesis of E. Coli in vitro"
Western Pharmacological Society Annual Meeting.
1988
"Melanoma Management"
Department of Surgery Grand Rounds, San Joaquin Memorial Hospital.
"The Phenomenon of Multiple Drug Resistance"
University of California, Davis, 10th Annual Surgical Postgraduate Course.
"Chemotherapy and Multi-Drug Resistance"
Sutter Memorial Hospital Department of Surgery Rounds.
"Management of Melanomas"
San Joaquin General Hospital Lecture Series.
"Skin and Melanoma".
Cancer Management Course, American College of Surgeons Commission on Cancer.
1989
"Approach to Retroperitoneal Tumors"
University of California, Davis, 11th Annual Surgical Postgraduate Course.
"Principles of Surg Oncol"
University of California, School of Veterinary Medicine Course. Current Topics in Veterinary Oncology.
"Management of Malignant Melanoma"
University of California, Davis Medical Center, Dermatology Grand Rounds,
"Use of Ultrasonic Dissector for Hepatic Surgery"
University of California, Davis, Surgery Grand Rounds.
1990
"The Use of Octreotide in the Treatment of Paraneoplastic Syndromes"
University of California, Davis, 12th Annual Surgical Postgraduate Course.
"Principles of Surg Oncol"

University of California, School of Veterinary Medicine course "Current Topics in Veterinary Oncology."

"Management of Malignant Melanoma"
San Joaquin General Hospital Lecture Series, Stockton, CA.

"Imaging of Tumors Using Radioactive-Tagged Monoclonal Antibodies"
University of Vermont, Surgical Grand Rounds.

"Measuring Ionized Calcium in Living Cells"
University of Vermont, Surgical Research Conference.

1991

"Clinical Update: mob-based Imaging Agents in Nuclear Medicine"
Society of Nuclear Medicine Meeting Cincinnati OH.

"Use of Monoclonal Antibodies for Diagnosis and Therapy of Cancer"
University of California, Davis, 13th Annual Surgical Postgraduate Course

"Intraoperative Detection of Ovarian and colorectal Cancer Using the Oncoprobe" Frontiers in Nuclear Medicine Symposium: Diagnostic uses of Radiolabeled Monoclonal Antibodies. American College of Nuclear Physicians and U.S. Department of Energy, Office of Health and Environmental Research, Office of Energy Research, Washington, D.C.

"Immunoscintography for Colorectal Cancer"
Gastroenterology Rounds, Medical Center Hospital of Vermont, September.

"Radiolabeled Monoclonal Antibodies for Diagnosis of Colorectal Cancer"
Hematology/Oncology Rounds, Vermont Cancer Center.

"Clinical Utility of Immunoscintigraphy in the Management of Ovarian Cancer"
Vermont Regional Cancer Center.

"The Use of Monoclonal Antibody for Detection of Ovarian and Colorectal Cancer"
The Nuclear Medicine Technologists Grass Roots Group Meeting, November.

"Monoclonal Antibody Imaging of Colorectal Cancer"
Surgical Grand Rounds, Medical Center Hospital of Vermont, Burlington, VT, December.

1992

"Breast Cancer:Controversial Aspects of Surgery and Radiotherapy"
Postgraduate course:Current Concepts and Controversies in Surgery
Department of Surgery, University of Vermont
Stowe, VT. February

"Tumors of the Liver: Surgical Management"
Current Concepts and Controversies in Surgery,
Stowe, VT. February

"Intraoperative Autotransfusion in Hepatic Resection for Malignancy: Is It Safe? "
Society of Surgical Oncology
New York, NY. March

"Tamoxifen Chemoprevention Trial: Breast Cancer"
Vermont Chapter American College of Surgeons. May

"Monoclonal Antibody Study - Imaging for Colorectal Cancer"
Educational Conference, MCHV Coordinating Council
Burlington, VT. August

"Thyroid Pathology and Physiology"
Residents Conference, University of Vermont College of Medicine
Burlington, VT. September

"Parathyroid Pathology and Physiology"
Residents Conference, University of Vermont College of Medicine
Burlington, VT. September

"Breast Cancer Diagnosis and Treatment"
Grand Rounds, MCHV, University of Vermont College of Medicine
Burlington, VT. September

"Breast Care Center"
Inservice, Department of Medicine, Division of Hematology/Oncology, University Health Center
Burlington, VT. September

"BCPT & Breast Care Center"
American Cancer Society, Vermont State Chapter, Inn at Essex
Essex Junction, VT. October

"Controversies in Breast Cancer Management"
Grand Rounds, MCHV, University of Vermont College of Medicine.
Burlington, VT. December

"The Breast Center"
MCHV Associates, Medical Center Hospital of Vermont
Burlington, VT. December

"Breast Cancer"
Associate Members of Education, Medical Center Hospital of Vermont
Burlington, VT. December

"Breast Cancer in Vermont"
The Lion's Club, Burlington, VT. December

1993

"Breast Cancer, Diagnosis and Medical/Surgical Interventions"
Association of Women's Health, Obstetric, and Neonatal Nurses (AWHONN), Medical Center Hospital of Vermont
Burlington, VT. January

"Breast Cancer Awareness."
North Country Hospital
Newport, VT. February

"Breast Cancer: Controversial Aspects of Surgery and Radiotherapy"
Postgraduate course: Current Concepts and Controversies in Surgery
Department of Surgery, University of Vermont
Stowe, VT. February

"Tumors of the Liver: Surgical Management"
Post graduate course: Current Concepts and Controversies in Surgery
Stowe, VT. February

"Breast Cancer"
Women's Legislative Caucus,
Montpelier, VT. February

"Current Management, Treatment, and Screening for Breast Cancer"
Postgraduate course: Current Concepts and Controversies in Surgery
Department of Surgery, University of Vermont
Stowe, VT. February

"BREAST HEALTH:Taking Care of You"
HEALTHBEAT/Spring into Health
Burlington, VT. April

"Cancer of the Breast"
Vermont Nurse Practitioner/Midwife Conference
S. Burlington, VT. April

"The Management of Breast Cancer"
OB & GYN Grand Rounds, University of Vermont
Burlington, VT. June

"Management of Colorectal and Ovarian "
Advances in the Diagnosis and Management of Colorectal and Ovarian
Puerto Vallarta, Mexico. April

"Breast Treatment and Management"
Vermont public radio
Burlington, VT. September

"Breast Cancer Detection and Tamoxifen Therapy"
WPTZ-TV
Colchester, VT. October

"Clinical Utility of Immunoscintigraphy in the Management of Colorectal Cancer"
Grand Rounds, McGee Women's Hospital
Pittsburgh, PA. September

"Intraoperative Detection of Radiolabeled Tissues"
Memorial Cancer Center
Chattanooga, TN. September

"Clinical Utility of Immunoscintigraphy in the Management of Colorectal Cancer"
Franklin General Hospital
Long Island, NY. September

"Hyperthermia: Past Experience and Future Prospects"
George M Hahn Symposium
Palo Alto, CA. September

"Intraoperative Detection of Radiolabeled Antibodies in Colon Cancer"
Postgraduate course: Gamma Probes for Intraoperative Detection of Radiolabeled Tissues, University of Vermont College of Medicine
Burlington, VT. October

"Gamma Probe-Guided Localization of the Sentinel Lymph Node in Malignant Melanoma Patients"
Postgraduate course: Gamma Probes for Intraoperative Detection of Radiolabeled Tissues, University of Vermont College of Medicine
Burlington, VT. October

"Introduction to Probes"
Postgraduate course: Gamma Probes for Intraoperative Detection of Radiolabeled Tissues
University of Vermont College of Medicine
Burlington, VT. October

"Surveillance, Diagnosis, and Treatment of Breast Cancer"
Vermont State Nursing Association
Burlington, VT. October

"Gamma Probe-Guided Lymph Node Localization in Malignant Melanoma "
Vermont Dermatological Society
Rutland, VT. November

"Treatment of Breast Cancer at the University of Vermont"
Lecture to staff of Medical Center Hospital of Vermont
Burlington, VT. December

"Adrenal Physiology"
Surgery Resident Conference
Medical Center Hospital of Vermont
Burlington, VT. December
1994

"Gamma Probe-Guided Radiolocalization of the Sentinel Lymph Node"
Sixth Annual Symposium/Diagnostic and Therapeutic Applications of Radiolabeled Antibodies and Receptors for Diagnostic and Therapeutic Purposes
Dept. of Nuclear Medicine, University of Miami
Key Biscayne, FL. January

"Intraoperative Detection of Radiolabeled Antibody Tissues in Colorectal Cancer"
Surgeon and Gastroenterologist Update Conference by University and Northside Hospitals of Georgia
Snowmass, CO. January

"Gamma Probe-Guided Localization of Lymph Nodes in Malignant Melanoma"
5th Annual Eastern Winter Dermatology Conference, University of Vermont
Stowe, VT. January

"Targeting Small Ligands"
Surgical Research Conference, University of Vermont
Burlington, VT. February

"Surgical Resection and Radiolocalization of the Sentinel Lymph Node in Melanoma and Breast Cancer Using a Gamma Probe"
3rd Annual Current Concepts and Controversies in Surgery
Department of Surgery, University of Vermont
Stowe, VT. February

"Immunoscintography in the Management of Colorectal Cancer"
American College of Surgeons Brooklyn/Long Island Chapter
Garden City, NY. March

"Surgical Resection and Radiolocalization of Sentinel Lymph Node in Breast Cancer Using a Gamma Probe"
Society of Surgical Oncology/47th Annual Cancer Symposium
Houston, TX. March

"Breast Cancer - What It Is and How It Is Treated"
Vermont Society for Medical Technology
Burlington, VT. April

"Gamma Probe for Intraoperative Detection of Radiolabeled Tissues"
Glens Falls Hospital
Glens Falls, NY. May

"The Current Diagnosis, Management, and Screening for Breast Cancer"
Postgraduate course: Family Practice Review Course, University of Vermont
Burlington, VT. June

"Targeting Breast Cancer with Small Ligands"
Signal Transduction Group, University of Vermont
Burlington, VT. September

"Minimal Access Surgery for Staging Breast Cancer"
Surgical Grand Rounds, Women's College Hospital
Toronto, Ontario, Canada October

"Minimal Access Surgery for Staging Breast Cancer"
CALGB Fall Group Meeting
Atlanta, GA November
"Minimal Access Surgical Staging of Melanoma"
Rutland Regional Medical Center
Rutland, VT. December
"Diagnosis and Management of Breast Cancer"
18th Annual Postgraduate Course in Obstetrics & Gynecology
University of Vermont
Burlington, VT. April
1995
"Sentinel Node in Presurgical Staging of Breast Cancer"
Nuclear Oncology: Advances in Diagnostic and Therapeutic Applications
University of Miami, Dept. of Radiology
Key Biscayne, FL. January
"Management of the Axilla"
Innovations in General Surgery
University of Miami School of Medicine
Miami, FL. January
"Radiolocalization Techniques for Chest Wall Disease & Bony Metastases"
Innovations in General Surgery
University of Miami School of Medicine
Miami, FL. January
"Gamma Probe-Guided Resection of Axillary Sentinel Node for Breast Cancer"
48th Annual Society of Surgical Oncology Symposium
Boston, MA. March
"Minimal Access Surgery for Staging of breast CA-Sentinel Node Concept"
Surgical Grand Rounds, Maimonides Medical Center
Brooklyn, NY. April
"Minimal Access Surgical Staging of Melanoma and Breast Cancer"
St. Joseph's Medical Center
Stockton, CA. July
"Breast Cancer Management"
Local chapter of the American Cancer Society.
Burlington, VT. September
"Workup of the Nonpalpable Breast Lesion"
Surgical Grand Rounds, MCHV, University of Vermont
Burlington, VT. September
"Breast Care Management Update"
ETV Public television: Across the Fence
Aired in October
Winooski, VT. September
"Minimal Access Surgical Staging of Malignant Melanoma and Breast Cancer"
Surgical Grand Rounds, Good Samaritan Hospital and Health Center
Dayton, OH. November

1996

"The Breast Care Center"
Development/Fletcher Allen Health Care
Medical Center Hospital of Vermont
Burlington, VT. January

"Minimal Access Surgery for Staging of Malignant Melanoma"
Eastern Winter Dermatology Conference
Dept. of Medicine, Dermatology Division, University of Vermont
Stowe, VT. January

"Breast Ultrasound"
5th Annual Current Concepts and Controversies in Surgery and Ultrasound Course
University of Vermont, Fletcher Allen Health Care, Department of Surgery
Stowe, VT. February

"Management of Patients w. Melanoma and Other Skin Cancers"
5th Annual Current Concepts and Controversies in Surgery and Ultrasound Course
University of Vermont, Fletcher Allen Health Care, Department of Surgery
Stowe, VT. February

"Minimal Access Surgical Staging of Malignant Melanoma"
New England Surgical Society 1996 Spring Meeting
Burlington, VT. March

"Selective Resection of Primary Lymph Nodes for Staging Breast Cancer"
National Surgical Adjuvant Breast and Bowel Project Group Meeting
Orlando, FL. March

"Intraoperative Ultrasonography"
Society of Surgical Oncology Minimal Invasive Surgery in Cancer Management Symposium
Atlanta, GA. March

"Intraoperative Detection of Sentinel Lymph Nodes"
Johns Hopkins Nuclear Oncology Course
Baltimore, MD March

"Primary Node Biopsy and Breast Cancer"
Postgraduate Course: The Multidisciplinary Breast Center
82nd Clinical Congress, American College of Surgeons
San Francisco, CA. October

"Intraoperative Ultrasound in the Excision of Nonpalpable Carcinoma of the Breast:A Preliminary Report"
Postgraduate Course: Ultrasound for the Surgeons
82nd Clinical Congress, American College of Surgeons
San Francisco, CA. October

"Lymph Node Mapping and Treatment for Breast Cancer"
University of Pennsylvania Medical Center
Philadelphia, PA November

"Targeting Lymph Nodes in Melanoma and Breast Cancer Patients"
Wayne State University School of Medicine
Detroit, MI December

"The Sentinel Node in Breast Cancer"
Deaconess Hospital
Boston, MA December

1997
"Sentinel Node Biopsies for Breast Cancer"
Meet the Professor Breakfasts
Society of Surgical Oncology 1997 annual meeting
Chicago, IL March
"Partial Lymphadenectomy for the Staging and Treatment of Breast Cancer"
American College of Surgeons '97 Spring Meeting/Postgraduate Course
"Controversial Issues in Cancer Management" pages 6-7
San Diego, CA April
"Limited and Targeted Node Biopsy for Staging Breast Cancer"
Postgraduate Breast Cancer Course
49th Southwestern Surgical Congress
Rancho Mirage, CA April
"Ultrasound for Surgical Residents '97"
University of Vermont
Burlington, VT May
"Sentinel Lymph Node Analysis: Can this be done percutaneously?"
"Breast Imaging and Intervention into the 21st Century: As Multi-Disciplinary Challenge," The Sally Jobe Breast Centre
Carlsbad, CA September
"New modalities in breast imaging"
"Breast Imaging and Intervention into the 21st Century: As Multi-Disciplinary Challenge,"
The Sally Jobe Breast Centre
Carlsbad, CA September
"Axillary Staging of Breast Cancer using the "Sentinel" Node Procedure "
Vermont Cancer Center Symposium
Sheraton Conference Center
So. Burlington, VT November
1998
"Sentinel Node in Breast Cancer-Multicenter Results"
7th Annual Current Concepts and Controversies in Surgery
Topnotch at Stowe Resort and Spa
Stowe, VT February
"Q & A/Discussion of Live Cases"
Breast Imaging and Intervention into the 21st Century:A Multi-Disciplinary Challenge
The Sally Jobe Breast Centre
Ocean Reef Resort
Key Largo, FL February
"Breast Ultrasound: Radiologist or Surgeons?"
Breast Imaging and Intervention into the 21st Century:A Multi-Disciplinary Challenge
The Sally Jobe Breast Centre
Ocean Reef Resort
Key Largo, FL February
"Sentinel Lymph Node Analysis —"
Breast Imaging and Intervention into the 21st Century:A Multi-Disciplinary Challenge
The Sally Jobe Breast Centre
Ocean Reef Resort
Key Largo, FL February

"Clinical Breast Problem Solving"
Breast Imaging and Intervention into the 21st Century:A Multi-Disciplinary Challenge
The Sally Jobe Breast Centre
Ocean Reef Resort
Key Largo, FL February

"Sentinel Node Tricks - A 'how-to' Workshop"
Breast Imaging and Intervention into the 21st Century:A Multi-Disciplinary Challenge
The Sally Jobe Breast Centre
Ocean Reef Resort
Key Largo, FL February

"New Possibilities for Minimally Invasive Breast Cancer Treatment"
Breast Imaging and Intervention into the 21st Century:A Multi-Disciplinary Challenge
The Sally Jobe Breast Centre
Ocean Reef Resort
Key Largo, FL February

"The Surgical Future of Breast Care"
National Consortium of Breast Centers, Inc. '98 Annual Meeting
Breast Center Development Conference
Atlanta, GA April

"Minimally invasive surgical staging of breast cancer"
19th Annual Gary P. Wratten Surgical Symposium
Mologne House, Walter Reed Army Medical Center
Washington, DC April/May

"Minimal surgical staging of breast cancer"
National Surgical Adjuvant Breast and Bowel Project
Grand Hyatt at Washington Center
Washington, DC June

"Sentinel node in breast cancer"
XVII UICC International Cancer Congress
Riocentro Convention Centre
Rio de Janeiro, Brazil August

"The use of intraoperative ultrasound to guided resection of impalpable breast lesion"
VIII Clinical Congress, Lebanon Chapter American College of Surgeons
Riviera Hotel
Beirut, Lebanon September

" Sentinel node biopsy in the surgery of primary breast cancer: the North American experience"
VIII Clinical Congress, Lebanon Chapter American College of Surgeons
Riviera Hotel
Beirut, Lebanon September

"Sentinel node biopsy: Breast"
American College of Surgeons 84th Annual Clinical Congress
Omni Hotel
Orlando, FL October

"Radioisotopes"
American College of Surgeons 84th Annual Clinical Congress
Omni Hotel
Orlando, Fl October

"Partial lymphadenectomy for staging breast cancer"
Elliot Hospital and Catholic Medical Center
Manchester, NH October
"NSABP B-32 Sentinel Node Clinical Trial"
Surgical Grand Rounds
Lankenau Hospital
Wynnewood, PA October
"The Sentinel Lymph Node in Breast Cancer"
Surgical Grand Rounds
The Breast Center at Mercy
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"Gamma probes for detection of radiolabeled tissues"
IEEE Nuclear Science Symposium
Westin Harbor Castle
Toronto, Ontario, Canada November
"NSABP B-32: A randomized, Phase III clinical trial to compare sentinel node resection to conventional axillary dissection in clinically node-negative breast cancer patients"
NSABP Group Meeting
Orlando, FL December
1999
"Research on Small Ligands"
Breast Care Affinity Group Meeting
University of Vermont
Burlington, VT January
"Sentinel node evaluation for breast cancer"
17th Annual Conference of Clinical Hematology and Oncology 1999
Scripps Clinic
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"Gamma probe-guided sentinel lymphodonectomy"
12th International Consensus Symposium: Sentinel Lymphonodectomy in Cutaneous Malignancies
Dept. of Dermatology and Allergology, Essex Pharma GmbH
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"The lymphatic system:the emergence of the sentinel node concept"
1st International Congress on the Sentinel Node in Diagnosis and Treatment of Cancer
Amsterdam, the Netherlands April

Co-Chair:
1) "Meet the expert" breakfast session Breast Cancer
2) Plenary sessions
1st International Congress on the Sentinel Node in Diagnosis and Treatment of Cancer
Amsterdam, the Netherlands April
3) Free paper session "Breast Cancer"
1st International Congress on the Sentinel Node in Diagnosis and Treatment of Cancer
Amsterdam, the Netherlands April
"Imaging diagnostic and sentinel node biopsy"
12th International Symposium of Foundation for Promotion of Cancer Research
Tsukuji, Tokyo, Japan April

Chairperson:
"Surgery and post-surgical follow-up"
12th International Symposium of Foundation for Promotion of Cancer Research
Tsukuji, Tokyo, Japan April
Lecturer:
Lymphatic Mapping Course
University of California San Francisco
Mt. Zion Medical Center
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"B-32 Trial"
Competitive renewal of our NCI grant
NSABP
Pittsburgh, PA September
"How to do it - radiocolloid alone"
American College of Surgeons Clinical Congress
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Panelist:
"Management of the axilla": The Role of the Surgeon in the Management of Breast Cancer
American College of Surgeons Clinical Congress
San Francisco, CA October
"Sentinel node evaluation for breast cancer"
23rd Annual Cancer Symposium for Physicians
Stevens Cancer Center, Scripps Memorial Hospitals
San Diego, CA October
"Sentinel lymph node biopsy for the detection of metastases"
National Oncology Forum
Chicago, IL October
2000
"History of sentinel node biopsy"
First Almanac Symposium: Sentinel Node Biopsy in Breast Cancer
University of Wales College of Medicine
Cardiff, UK January

"Sentinel node in breast cancer"
Miami Breast Conference
Loews Miami Beach Hotel
Coral Gables, FL March
"Ultrasound-guided resection of nonpalpable lesions"
Miami Breast Conference
Loews Miami Beach Hotel
Coral Gables, FL March
"Sentinel node in breast cancer and melanoma"
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"Intraoperative ultrasound-guided resection of nonpalpable lesions"
XI International Congress of Senology
Cancun, Mexico

"NSABP (B-32) trials: past, present and future"
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Updated 6/6/00

Introductory Statement and General Investigational Plan

We are requesting an IND for a process which we call "in vivo screening of phage-displayed random peptide libraries", which we will refer to in this package as "in vivo RPL screening".

The broad objective is to identify small molecules that bind specifically to tumor cells or to endothelial cells lining blood vessels specifically supplying tumor tissue. Such molecules can be conjugated to cytotoxic agents or immunomodulatory agents in order to eradicate tumor tissue without harming normal cells. In other words, this product will be used in the development of novel and effective therapeutic agents for cancer patients.

The RPLs to be used for this project have been constructed in a phage-display system. Phage-displayed are particularly powerful in that the peptides are physically linked to their encoding DNA. Because DNA is easily amplified for sequencing, one binding peptide out of millions can be determined. The RPL is made using filamentous phage which infect and multiply in *E. coli*. Each phage particle has five copies of a minor coat protein (pIII) located at one end. Random synthetic DNA is inserted into the gene coding for pIII so that the foreign DNA is expressed at the free N-terminus of pIII as random peptides. Up to 5 copies of each peptide are physically "displayed" by each phage particle. Each phage particle displays a different peptide and is easily amplified in *E. coli*. After amplification the phage DNA is sequenced to deduce the identity of the displayed peptide. The small size of the library particles allows manipulation of millions of different potential binding units in a few microliters.

The route of administration will be intravenous. The broad objectives are to develop small molecule ligands against biological targets for the purposes of anticancer therapy. The planned duration will be 12 to 24 months.

Dr. Hans Ochs, an immunologist who is one of the pioneers of using phage ϕX174 for antibody response analysis, is a collaborator on this project. Dr. Ochs's group has had an IND from the FDA (IND number BB-IND 714, ϕX174, Dr. Hans Ochs, University of Washington, Seattle, WA) for over 20 years for this procedure and has performed extensive studies with bacteriophage injected IV in humans. It has been used safely in over 3000 patients to monitor antibody responses with only rare adverse reactions in patients with unusual genetic immune deficiencies. Investigations were carried out by his group to detect phage DNA incorporation into eukaryotic cells and was found only transiently in lymphocytes.

To our knowledge this biological drug has not been previously withdrawn

Plan for investigation: We propose the use of in vivo RPL screening to identify small peptide ligands to tumor-specific molecular targets in patients with breast cancer or malignant melanoma. In future studies, these peptides will be modified if necessary to optimize *in vivo* stability, coupled to cytotoxic or other therapeutic agents, and used to mediate the specific destruction of cancer cells.

Rationale: A major problem in the treatment of cancer is that present therapies lack specificity for tumor cells and are extremely toxic to normal cells. The development of therapies with high specificity for tumor are an utmost priority in cancer research. Exciting progress has been made in the elucidation of key molecules found specifically overexpressed or underexpressed in

cancer cells. However, effective ways to exploit these tumor-associated targets for therapy have not yet been developed. A means to specifically direct therapeutic agents to these defined molecular differences is critically needed. Large libraries of small compounds are a rich source of small ligands which may target tumors. These libraries which consist of millions or even billions of different peptides have been constructed by our laboratory to identify small peptide ligands which will specifically bind to tumor cells and not to normal cells.

Other advantages of peptide ligands for tumor targeting are that they can be easily synthesized in the large amounts needed for clinical use, their chemistry is well known, and conjugation methods are routine. Peptides are very promising targeting agents because they can potentially bind targets with the same exquisite specificity as antibodies, and are likely to have far more favorable pharmacokinetics. Alternatively, peptide motifs can be used to direct other agents which have therapeutic activity, such as cytotoxic drugs, immune modulating agents, ribozymes, and gene therapy delivery systems such as liposomes or viral particles, to a specific molecular target. Peptides can also potentially target imaging agents for diagnostic purposes.

In vivo RPL screens in humans offer several potentially critical advantages over *in vitro* screening including: 1) Tumor targets will be in their native conformation with all their human post-translational modifications. 2) Inherent selection of peptides that recognize *specific* targets due to efficient "subtraction" of library clones which bind to normal tissue during exposure of the injected library to the entire body. 3) Inherent selection of *only* peptides which are stable *in vivo*. 4) Inherent targeting of *only* targets which are stable *in vivo* and which are capable of binding ligands *in vivo*. 5) Purification or prior knowledge of particular targets is not necessary. 6) Potential elucidation of novel tumor targets. Targets are not influenced by immunogenicity as with targets defined only by monoclonal antibody development.

General approach: Eligible patients will have advanced breast cancer or malignant melanoma. Blood levels of anti-bacteriophage antibodies will be determined by ELISA prior to administration of the bacteriophage library.

Patients will be admitted to the University of Vermont General Clinical Research Center (GCRC). An intravenous line will be placed. Baseline vital signs (blood pressure, pulse, temperature, and respiratory rate) will be determined before infusion of phage, every 15 minutes during infusion and for 2 hours after infusion. There is the risk of an allergic reaction, including an anaphylactic reaction, associated with the administration of the phage library. Therefore, the patient will be closely monitored and personnel skilled in handling allergic reactions will be present during infusions and all equipment for handling such reactions will be immediately available. The likelihood of adverse reactions is low since a similar strain of bacteriophage has been injected safely into several thousand patients. The small number of individual molecules of each type of peptide which will be injected are not expected to result in toxicity.

The peptide-phage to be injected will be prepared and tested for sterility and pyrogenicity according to FDA standards prior to administration. Before phage injection, one sample of tumor tissue from the patient will be biopsied, snap frozen and sixty slides will be prepared for later testing of selected clones for tumor binding affinity. The library will be diluted in 250 ml saline and infused intravenously over 10 minutes. Small amounts of tumor tissue will be removed in the procedure room of the GCRC at time points of 10 minutes or 24 hours post infusion. Standard surgical technique typical for performing a surgical biopsy will be utilized for the biopsy procedures. It is intended that the biopsies be as small as possible (one gram or less).

The tumor tissue will be ground and *E. coli* will be added to amplify phage. Phage will be eluted from tumor cells and amplified. Phage will be amplified and quantified by titering, with results available within 12 hours. Amplified phage will be prepared as was described above for initial infusion and will be reinjected as soon as possible. The harvest and amplification sequence will be repeated a maximum of 2 times for a maximum of three screenings in one patient. Screening will ideally be completed in less than 7 days to avoid a patient Ab response to phage. Throughout the screening process patients will be carefully evaluated for adverse reactions.

Peptides displayed by phage isolated from tumor tissue will be routinely analyzed for both consensus amino acid sequences and tumor-binding. Any consensus sequences identified from phage eluted specifically from the tumor tissue will be candidates for tumor-specific peptides. Tumor specific binding will be assessed by IHC on tissue sections of tumor harvested before phage injection. Binding to normal tissue will be assessed by IHC on normal, quick frozen breast tissue excised at the same time as the first tumor biopsy (before phage injection) and on a large panel of 32 different normal human tissues. IHC with anti-transferrin receptor mAb will be used as a positive control to assure tissue and assay reliability.

Promising peptides will be synthesized and tested for tumor specificity. Peptide binders we identify by whole body screens, almost by definition, are likely to be stable in serum and generally stable *in vivo*, a major advantage to this technique.

In future studies, we will evaluate the efficacy of peptide-therapeutic conjugates. In addition to attaching standard cytotoxic drugs to the ligands, in separate experiments we will attach immunogenic peptides, perhaps one to which most people have already been immunized against. The binding of a molecule bearing an immunogenic peptide to the tumor cell surface may stimulate the immune system to eliminate the tumor cell. Thus, instead of using dangerous chemicals or radiation, we may be able to direct the body's own immune system to more naturally eradicate tumor cells.

The estimated number of patients will be twenty. The most severe possible risk is allergic reaction. This will be monitored closely and on immediate standby will be full support methods to treat any potential reaction.

Bacteriophage are known to specifically infect only bacteria, and each bacteriophage strain infects only a very narrow range of bacterial species. The possibility of bacteriophage "infecting" eukaryotic cells in any way is highly unlikely.

In Vivo Selection of Ligands for Targeted Therapy

PRINCIPAL INVESTIGATOR
David N. Krag, MD
309C Given Building
University of Vermont
Tel: 656-5830
Fax: 656-5833
Email: dkrag@salus.med.uvm.edu

OPERATIONS CENTER
Vermont Cancer Center
24C Hills Science Building
University of Vermont
Tel: 656-4270
Fax: 656-1987

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1.0 INTRODUCTION

A major problem in the treatment of breast cancer is that present therapies lack specificity for tumor cells and are extremely toxic to normal cells. The development of therapies with high specificity for tumor is an utmost priority in breast cancer research. Exciting progress has been made in the elucidation of key molecules found specifically overexpressed or underexpressed in breast cancer cells. However, effective ways to exploit these tumor-associated targets for therapy have not yet been developed. A means to specifically direct therapeutic agents to these defined molecular differences is critically needed. Many high molecular weight targeting agents, antibodies in particular, have been identified which are specifically directed against tumor-associated molecules. However, coupled to cytotoxic agents or alone, the performance of antibodies (Abs) or Ab fragments in clinical trials has been disappointing [22, 45, 46, 48, 49]. Results with Herceptin, an antibody against the clinically important breast cancer target ErbB2, have been more promising than most antibody trials, and confirm the value of ErbB2 as a target. However, the clinical responses with Herceptin are far from ideal [15, 75]. Failure of antibodies in the clinic is likely due to the unfavorable pharmacokinetics, lack of tumor penetration, and immunogenicity of molecules this large, as well as their non-specific uptake by the reticuloendothelial system [16, 33, 41, 57]. Smaller single chain Fv (sFv) Ab fragments have been developed with high affinity to ErbB2 [70] and it will be interesting to see how these molecules perform in the clinic. However, the vast majority of effective drugs are of much lower molecular weight than sFvs (25kD) and the discovery of smaller tumor-specific ligands would be extremely valuable. As has become apparent with the Herceptin trials, and also in Judah Folkman's promising work with antiangiogenesis factors, large proteins are also difficult to synthesize in amounts necessary for clinical use. Furthermore, many of the most promising cancer-specific targets are intracellular or intranuclear. Antibodies are not likely to be effective against these intracellular targets. Because of this disappointing progress in tumor-targeted therapy over the past decades, it is clear that dramatically innovative approaches are needed.

Our lab is attempting to identify novel *small* ligands (1-2 kD) that bind specifically to tumor cells. Small ligands may have therapeutic activity alone, as does Herceptin, presumably by inhibiting a target molecule that actively plays a role in carcinogenesis. Small ligands can also be coupled to cytotoxic agents and used to mediate the specific destruction of tumor cells, even if their target molecules do not play an active role in cancer progression, as long as they are specifically present on the tumor (or on blood vessels specifically supplying tumor.) Ligands much smaller than antibody fragments may have important advantages in targeted therapy including improved tumor to non-tumor uptake ratios, better penetration of solid tumors, and non-immunogenicity. Small molecules are also easier to synthesize in the large amounts necessary for clinical use, are less likely to interfere with the effects of conjugated cytotoxic drugs, and may have improved specificity as there is less surface to interact non-specifically with other body components.

Large libraries of small compounds are a rich source of small ligands that may target tumors. Several types of these libraries, which consist of millions or even billions of different peptides, oligonucleotides, or synthetic molecules, have been constructed and used to isolate small ligands or lead compounds to many targets. The construction of libraries like these and their use in the identification of specific ligands, known as combinatorial technology, has revolutionized the field of drug discovery [28]. This proposal describes the use of this technology to identify small *peptide* ligands that will specifically bind to tumor cells and not to normal cells.

Although peptides have traditionally been discounted as potential therapeutics due to an assumption of their instability *in vivo*, peptides can form an almost infinite number of shapes and are exactly what nature uses to specifically target molecules both intracellularly and extracellularly. Many peptides have important biologic functions and potent *in vivo* activities. Furthermore, the exciting work of Ruoslahti et al [2, 60, 61, 65] has demonstrated that many peptides are stabile enough in serum to home specifically to tumors and to various organs. Elegant

experiments from Affymax [18, 90] also demonstrated that, with minimal modification, peptides are capable of strong binding and effective agonist activity *in vivo* to cell surface receptors. Furthermore, small peptide ligands which are identified to tumor targets by *in vitro* screening, even if lacking *in vivo* stability, can be modified and/or used as a prototype in order to develop a small molecule which will be more stable and effective *in vivo*. We have already identified such a small peptide ligand to a potential tumor target, Grb2, and our collaborators have modified it chemically such that it retains binding activity in cell lysates [59]. Cyclic peptides are more stable *in vivo* and are often more selective for their targets. Many peptide libraries, including those used in our lab, are biased for cyclic peptides. D-amino acids, non-natural amino acids, and pseudo-peptide bonds may also confer greater *in vivo* stability. A novel and intriguing method of obtaining mimetics that may be more stable *in vivo* involves using a D-amino acid synthesized target. Screening with the D-amino acid target can result in the identification of D-amino acid peptide ligands to natural L-amino acid targets [71]. Peptide structures can also be readily determined by NMR and used to model peptidomimetics that may be more stable *in vivo*. The structure of peptide ligands can also provide important information about the structure of both their receptor targets and the natural ligands of those targets. Identification of peptide ligands can also facilitate the discovery of natural ligands to orphan receptors such as ErbB2. Other advantages of peptide ligands for tumor targeting are that they can be easily synthesized in the large amounts needed for clinical use, their chemistry is well known, and conjugation methods are routine.

Peptides and peptidomimetics are very promising targeting agents because they can potentially bind targets with the same exquisite specificity as antibodies, and are likely to have far more favorable pharmacokinetics. Peptides can have direct agonist or inhibitory activity on therapeutic targets. In another project, our lab is attempting to identify peptides that directly inhibit the dimerization of ErbB2, an effect that may result in therapeutic activity. Alternatively, peptide motifs can be used to direct other agents that have therapeutic activity, such as cytotoxic drugs, immune modulating agents, ribozymes, and gene therapy delivery systems such as liposomes or viral particles, to a specific molecular target. In a remarkable recent report, a short peptide sequence was grafted onto a 41kD protein capable of inhibiting growth factor receptor signaling [69]. The peptide allowed the protein to penetrate the cell membrane and to be delivered into the cytosol from the extracellular environment without detectable proteolysis. The delivered protein was successful in inhibiting growth factor signal transduction. Peptides can also potentially target imaging agents for diagnostic purposes.

Using a combinatorial approach, large random peptide libraries (RPLs) have been constructed in several systems. The RPLs described in this proposal have been or will be constructed in a phage-display system [19, 21, 83]. Phage-displayed and other biological RPLs are particularly powerful in that the peptides are physically linked to their encoding DNA. Because DNA is easily amplified for sequencing, one binding peptide out of millions can be determined. Phage-displayed libraries are made using filamentous phage that infect and multiply in *E. coli*. Each phage particle has five copies of a minor coat protein (pIII) located at one end. Random synthetic DNA is inserted into the gene coding for pIII so that the foreign DNA is expressed at the free N-terminus of pIII as random peptides. In this system, up to 5 copies of each peptide are physically "displayed" by each phage particle. (Another commonly used phage-display system employs the major coat protein and displays several hundred peptides per particle.) Each phage particle displays a different peptide. A phage particle bearing a peptide which bonds to a target can be isolated using affinity selection and is easily amplified in *E. coli*. After amplification the phage DNA can be sequenced to deduce the identity of the displayed peptide. The small size of the library particles allows manipulation of millions of different potential binding units in a few microliters.

Phage-displayed RPLs have been used by our lab and others to isolate small ligands, some with nanomolar and even picomolar affinity, to a large variety of targets including several potential tumor targets and other clinically important targets [2, 18, 60, 61, 65, 90]. One example of the use of small peptides (8 and 12 mer) in targeting tumors has been reported by Renschler et al. [66, 67]

who used phage displayed RPLs to identify peptides that bind to the antigen binding receptor of B-lymphoma cells and induce apoptosis *in vitro*. Most of these ligands have been identified using *in vitro* screening techniques: binding purified target protein to a matrix, incubation of the immobilized protein with the peptide-phage library, washing away non-specific binders, elution of specifically bound phage, followed by phage amplification and DNA sequencing to determine the identity of the peptide responsible for binding activity.

This proposal describes the use of phage-displayed RPL technology to identify small peptide ligands to breast cancer-specific targets by *in vivo* screening in breast cancer patients. In future studies, these peptides will be modified if necessary to optimize *in vivo* stability, coupled to cytotoxic or other therapeutic agents, and used to mediate the specific destruction of breast tumor cells. Proof of concept for our project is found in several exciting recent reports [2, 18, 60, 61, 65, 90], one which describes *in vivo* screening of RPLs similar to ours in mice bearing human tumor xenografts and the identification of peptides which home specifically to tumor blood vessels. Administration of peptide-doxorubicin conjugates to tumor-bearing mice resulted in a marked decrease in doxorubicin toxicity, selective tumor destruction, and excellent animal survival [2, 4]. The same group has also identified peptides that bind preferentially *in vivo* to at least 10 different organs [61, 65], further demonstrating the powerful ability of small peptides to home to specific molecular "addresses" in the body. As mentioned above, Affymax has also identified peptides from libraries similar to ours that bind with high affinity *in vivo* to clinically important cell targets. These important findings have introduced a whole new field of exploration in the search for more specific and effective cancer therapeutics.

We are in a unique position to perform the novel *in vivo* RPL human screening experiments described in this proposal. We have extensive experience with phage-RPLs and have successfully developed binders to several potentially clinically useful targets. The PI has extensive clinical experience in the IV injection of experimental compounds such as radiolabeled monoclonal antibodies and the intratumor injection of technetium colloid materials. In vivo RPL screening experiments [2,4,61,65] are certainly among the most intriguing and novel in cancer research today. We feel it is important and urgent to pioneer similar techniques in humans, as the identification of ligands that recognize mouse endothelial cells may not be relevant to treatment of human breast cancer.

In vivo RPL screens in humans offer several potentially critical advantages over *in vitro* screening including:

1) Tumor targets will be in their native conformation with all their human post-translational modifications. Screening targets in their native *in vivo* conformation may be especially important in light of the following: host endothelial targets may be among the most promising "tumor" targets available [10-12, 27, 37, 60] [4] and "the phenotype of endothelial cells is unstable and likely to change when the cells are removed from their microenvironment [3, 8, 65]." Therefore, IV injection of RPLs *in vivo* may not only be the *optimal* method of presenting these important endothelial targets to the library- it may be the *only* effective method. Host targets, which are genetically stable and homogenously present on target tissues, are particularly attractive due to the heterogenous nature of most cells of a tumor, which may be difficult to target with even a combination of drugs. However, targets located directly on tumor cells are also most advantageously presented in their native conformation.
2) Inherent selection of peptides that recognize *specific* targets due to efficient "subtraction" of library clones which bind to normal tissue during exposure of the injected library to the entire body. Efficient removal of library clones that bind normal tissue is essential to the recovery of tumor-specific clones. It would be difficult to "subtract" all clones that bind to every possible normal tissue by *in vitro* subtraction methods, as this would require having fresh tissue samples from dozens of human tissues harvested from a cadaver. Subtraction with fixed tissues may not give satisfactory results since all targets do not retain native conformation during tissue fixation. Obviously, *in vitro* subtraction with all possible

normal tissues, fresh or fixed, would be impossible to do with the normal tissue of the same patient being screened. Rajotte *et al.* [65] noted how their *in vivo* screening method "surprisingly...consistently yielded tissue-specific homing peptides" and attribute this success to efficient whole body "elimination of nonspecific phage." Further evidence that *in vivo* screening methods may be more effective than *in vitro* methods in the selection of specific ligands to cell surface targets is supplied in an interesting report by Barry et al [5]. Using whole cells to pan *in vitro*, peptides were identified which bound to many cell types rather than peptides that bound specifically to the cell type used for screening.

3) Inherent selection of *only* peptides that are stable *in vivo*.
4) Inherent targeting of *only* targets that are stable *in vivo* and which are capable of stably binding ligands *in vivo*.
5) Purification or prior knowledge of particular targets is not necessary.
6) Potential elucidation of novel tumor targets. Targets are not influenced by immunogenicity as with targets defined only by monoclonal antibody development [5].

Screening whole cells or cell extracts in vitro can also achieve the latter two advantages; however, many of the important advantages will be lost using this technique. Similarly, while direct injection of tumor with RPLs for screening is certainly possible, the important advantage of whole body elimination of normal tissue binding clones will be lost, and the chance of exposing the library to the important apical surface of endothelial cells will be greatly decreased. Systemic injection may well be the most effective way to identify ligands with a good tumor/control tissue-binding ratio. For example, in previous in vivo screening experiments, a peptide with a RGD motif binds integrins three orders of magnitude better than an NGR peptide. However, the NGR peptide had a tumor/control tissue homing ratio three times better than that of the RGD peptide [2].

The following proposal will extend *in vivo* screening techniques in the following ways to optimize its success in identifying small tumor-specific ligands in humans:

1) Identification of *human* targets. Ligands to mouse endothelial targets are not likely to be as effective or effective at all for human targets. In the previous *in vivo* tumor screening study in mice, many of the tumor-homing peptides contained an RGD amino acid motif, as did one of the two peptides assessed for their ability to direct doxorubicin to tumors [2]. Although integrins and related receptors are very promising tumor targets, and many integrin binding sequences contain an RGD motif, the regions flanking RGD are critical to the specificity of integrin binding. Optimal flanking regions of these integrin binding peptides are not likely to be identical for the recognition of both mouse and human integrins on newly developed endothelial cells, as mouse and human integrins are not identical as assessed by GCG analysis. Peptides identified by human *in vivo* screenings are far more likely to yield specific and high affinity binders to human tumors.
2) Identification of targets in a realistic setting. The mouse model was a xenograft model, and did not involve mouse tumor. This artificial model may yield results not applicable to the eradication of natural tumors.
3) By using a large panel of libraries that offer a variety of structural contexts for peptide presentation, we believe we may isolate peptides that bind to other promising breast cancer targets as well as to tumor-associated integrins. It is likely that a successful therapeutic regimen for advanced cancer will require a "cocktail" of anti-cancer compounds. Targeting a single cancer target is not likely to effect a long-term favorable therapeutic response. In addition, there is evidence that other sequences may bind as tightly and more specifically than RGD sequences to integrins [9]. It is also possible that RGD and NGR peptides [2] may bind to too many targets to be specific enough. Even low affinity binding can have significant biological consequences, particularly with avidity effects, as is the case with integrin binding to fibronectin. Furthermore, a large panel of libraries which present a vast number of peptides presented in a variety of structural contexts is much more

likely to yield a high affinity binder to any given target [7]. We will also employ several innovative RPL technical improvements, developed by both our lab and Affymax.

4) We will attempt to identify peptide ligands to endothelial targets by harvesting tissue within 10 minutes after library injection. However, by harvesting tissue 24 hours after library injection, we will also attempt to identify peptides that bind directly to tumor cells or are taken up by tumor cells.
5) We will attempt to assay peptide binding directly rather than via competition with peptide-phage as done in previous *in vivo* screening experiments. This will allow us to more accurately determine the degree of specificity by immunohistochemical analysis of peptides on tumor tissues and a large panel of normal tissues.
6) By performing multiple screens in one person, rather than serially as was done with mice, we may determine which targets are *unique* to a particular individual. (Important *generic* targets will be identified by similarities in consensus sequences identified from different patients in whom complete multiple screenings were performed, or by serial screens between different patients.) Even though screens will be initially performed on patients with advanced disease, it is very conceivable to establish a profile of ligands against the majority of newly diagnosed patients with breast cancer immediately prior to definitive surgery. This would allow design of systemic adjuvant therapy most appropriate to each patient.
7) Very importantly, we will carefully investigate toxicity during phage injections and screenings in both animals and humans. Toxicity during *in vivo* RPL screening has not previously been studied.
8) We will test several screening designs, including multiple screenings in one patient to determine the safest and most effective screening protocol.

A large portion of the following proposal is designed to examine possible toxicity during *in vivo* screening. We do not expect the screening procedure to cause toxicity as bacteriophage have been injected intravenously in humans and even neonates for over 30 years in approximately 3000 patients with essentially no side effects. This is extensively reported in the literature [13, 36, 58, 62, 85] Phage are injected into humans IV routinely for analysis of antibody responses.

There has also been extensive use of over 250 strains of bacteriophage, including 39 that infect *Escherichia* bacteria, which were administered orally or locally for treatment of infection [76-82]. Not only were "side effects" described as "extremely rare" (3 allergic responses out of 138, with no prior endotoxin testing), the phage treatments were often effective in eliminating the bacterial infection.

Bacteriophage are known to specifically infect only bacteria, and each bacteriophage strain infects only a very narrow range of bacterial species. In addition, it has long been known that ssDNA is expressed poorly or not at all in mammalian cells. The presence of phage DNA in human cells was examined in the antibody analysis studies mentioned above several months after injection and none was detected. Therefore the possibility of bacteriophage "infecting" eukaryotic cells in any way is highly unlikely. Nevertheless, we will carefully monitor this possibility by PCR.

Dr. Hans Ochs, an immunologist who is one of the pioneers of using phage ϕX174 for antibody response analysis, is an enthusiastic collaborator on this project. Dr. Och's group has had an IND from the FDA for over 20 years for this procedure. We will have his expertise available to us for consultations on human phage injection and he will provide us with his strain of phage for library construction if necessary. Not only has the work of Och's et al been extensively documented for non-toxicity, their experiments give us an accurate estimate of the time we have available to screen before we expect immune system interference. Our library is constructed in a strain of bacteriophage different than the strain used for antibody response analysis, although they both infect *E. coli* exclusively. However, filamentous phage injection is not likely to cause toxicity, as there have been numerous reports of the injection of filamentous phage into mice for

several other purposes including hybridoma development. There are several groups investigating the use of filamentous phage particles as potential vaccine delivery agents, with numerous preliminary studies in mice. At least one of these studies reports IV injection of a very large number ($2x10^{12}$) of phage particles with no toxicity reported, although toxicity was not explicitly addressed [32]. The mice survived at least 3 to 4 days when they were sacrificed in order to harvest spleen cells for hybridoma development. Our phage will also bear peptides, which may present complications particularly after amplification from tumor tissue, which will likely have normal tissue components. Therefore, the proposal has been carefully designed to detect any possible toxicity. We have had an active dialogue going on for several months with both the NCI and the FDA on this matter, and their input has shaped the present design. A pre-IND meeting with the FDA is scheduled for November 1998.

2.0 OBJECTIVES

Specific Aim: T*he safety of IV administration of phage RPLs in human patients with breast cancer will be established with both naïve libraries and amplified libraries.* These studies will be the equivalent of Phase I trials. Screenings will be performed three times over a time period of <14 days. Throughout the screening process patients will be carefully evaluated for adverse reactions. Peptides displayed by phage isolated from tumor tissue will be routinely analyzed for both consensus amino acid sequences and tumor-binding. Binding to normal tissue will be assessed by immunohistology on a large panel of 35 different normal human tissues. *Successful completion of this aim will establish the safety of in vivo phage RPL screening in humans and will result in the identification of peptides which bind specifically to breast tumor cells or to blood vessels specifically supplying tumor cells in human patients. Whole body in vivo screening experiments will result in the development of methods that may allow identification of novel tumor targets and greatly improved therapeutics.*

Hypotheses 1. Small peptides can be identified from RPLs that will bind specifically to human tumor cells and/or to blood vessels specifically supplying tumor by *in vivo* screening in human breast cancer patients. 2. One way of identifying these peptides, and perhaps the most efficient way, is to inject phage RPLs libraries into patients and to harvest specific peptide-phage directly from resected tumor tissue. 3. Small ligands that bind specifically to cancer cells will allow development of more effective cancer therapeutics.

3.0 PATIENT ELIGIBILITY CRITERIA

3.1 **Histologic documentation:** patients with metastatic or locally advanced primary or recurrent breast cancer that is not considered curable by conventional therapy.

3.2 **Prior Treatment:** No Limitations.

3.3 **Measurable Disease:**
Superficial cancer nodules or mass amenable to biopsy with minor surgery.

3.4 Age limitations: 30 – 70 years of age
Performance score: Karnofsky status ≥ 70
Life expectancy: ≥ 4 months
Non-pregnant

3.5 **Informed Consent:** the patient must be aware of the nature of his/her disease and willingly consent after being informed of the procedure to be followed, the experimental nature of the procedure, alternatives, potential benefits, side effects, risks, and discomforts.

3.6 **No previous or concurrent malignancy** is allowed, except inactive non-melanoma skin cancer, in situ carcinoma of the cervix, or other cancer if the patient has been disease-free for ≥ 5 years.

3.7 **No other serious medical illness,** other than that treated by this study, which would limit survival to < 4 months, or psychiatric condition which would prevent informed consent.

3.8 **No evidence of extensive pulmonary metastases** evidenced by chest roentgenogram within 60 days of the protocol.

3.9 **No clinical symptoms suggestive of brain metastases** unless ruled out by imaging studies.

4.0 Patient Registration

4.1 Authorized physicians or designees must fill out confirmation of registration sheet (Appendix II) fax into the Operations Center 656-4270 to obtain a patient number. Office hours are 8:00 to 4:30.

4.2 All patients will sign an approved informed consent that provides full disclosure of the procedure, rationale, plan, and risks.

4.3 At the time of registration, the investigator may be asked to respond to a list of questions related to the patient's eligibility for this protocol. The eligibility checklist is located in Appendix II

4.4 When a patient is removed from protocol (e.g., because of disease progression or drug-related toxicity), the Operations Center is to be called and given the reason for the patient's removal from the protocol and the date on which discontinuation of protocol occurred.

4.5 We estimate that we will study 20 patients.

5.0 Required Monitoring of Patients

Patients will be admitted to the General Clinical Research Center (GCRC). An intravenous line will be placed. Baseline vital signs (blood pressure, pulse, temperature, and respiratory rate) will be determined before infusion of phage, every 15 minutes during infusion and for 2 hours after infusion. The patient will be under constant supervision and equipment, medications, and personnel capable of treating allergic (including anaphylactic reactions) will be immediately available.

	Before Infusion	Every 15 Minutes During Infusion	Two Hours after Infusion
Blood Pressure	X	X	X
Pulse	X	X	X
Temperature	X	X	X
Respiratory Rate	X	X	X

Screening will ideally be completed in less than 7 days to avoid patient Ab response to phage [62]. Throughout the screening process patients will be carefully evaluated for adverse reactions.

6.0 Study Plan

Approximately 20 patients will be subjected in vivo screening in an attempt to identify peptides that home specifically to their tumor tissue. It is highly unlikely that cancer patients have

preexisting intact filamentous phage located in their tumor tissue. Nevertheless, as a control, biopsies from two patients, uninjected with phage, will be assayed for the presence of phage. In the experimental group, if phage clones isolated from tumor tissue after the third tissue harvest display a consensus amino acid sequence, it is highly likely that those peptide-phage are binding specifically to some component of the patient's tissue. An internal control will be to compare the sequences of phage clones eluted from the first tissue harvested (expected to be relatively random) compared to the last tissue harvested, as well as comparing clone sequences from peptide-phage isolated from different patients. Regarding the ability of this procedure to identify peptides which home specifically to tumors, we do not believe methods to minimize bias on the part of subjects, investigators, and analysis are necessary, as the measurements to be made: the number of phage eluted from the tumor, and the sequence of the peptides displayed by phage clones eluted from tumor, are objective and not subject to human bias. Regarding the possible side effects caused by this procedure, bias will be minimized by having all procedures performed in the University of Vermont General Clinical Research Center (GCRC). The staff at the GCRC are expert in observation of patients during experimental protocols and are not supervised by the PI of this investigation.

Patients will be admitted to the GCRC. An intravenous line will be placed. Baseline vital signs (blood pressure, pulse, temperature, and respiratory rate) will be determined before infusion of phage and every 15 minutes during infusion and for 2 hours after infusion. The patient will be under constant supervision and equipment, medications, and personnel capable of treating allergic (including anaphylactic reactions) will be immediately available. Anti-bacteriophage antibodies will be determined by ELISA prior to administration of the bacteriophage library. Based on previous studies [13, 36, 58, 62] very few individuals have pre-existing bacteriophage antibodies and it is expected that antibacteriophage antibodies will develop subsequent to phage library administration similar to that demonstrated for ϕX174. Since positive titers are expected and serial administration of bacteriophage has resulted in only rare reversible side effects, subsequent titer levels will not be obtained for this protocol. In the event that the number of phage recovered from the tumor specimens harvested after the second phage library administration are extremely low, titer levels of anti-phage antibodies will be determined in order to determine if antibodies are the cause of low phage recovery.

Ideally, we will screen our RPL(s) three times in the same patient. In the unlikely event of toxicity with naïve libraries, no further pans will be performed. In the event that only amplified phage show evidence of toxicity, no further pans will be performed.

A phage displayed RPL pool will be prepared and tested according to FDA standards as discussed below in "Pharmaceutical Information/Preparation". Before phage injection, one sample of tumor tissue from the patient will be biopsied, snap frozen and sixty slides will be prepared for later testing of selected clones for tumor binding affinity. The library will be diluted in 250 ml saline and infused intravenously over 10 minutes into a breast cancer patient. Initially $10^{9\text{-}10}$ pfu will be injected as that amount was found to be completely non-toxic to humans in similar studies [62]. Higher numbers of phage, up to $10^{14\text{-}16}$ or more may be necessary if we fail to harvest phage from a small amount of resected tumor. An appropriate dose will be one in which peptide-phage are isolated from small tumor biopsies, and preferably, one in which peptide-phage isolated from tumor tissue display homologous amino acid sequences. Small amounts of tumor tissue will be biopsied in the procedure room of the GCRC at time points of 10 minutes and 24 hours post infusion. Standard surgical technique typical for performing a surgical biopsy will be utilized for the biopsy procedures. It is intended that the biopsies be as small as possible on the order of 1.0 gram of tissue. The method of biopsy will be either incisional, excisional, or core depending on the location of the tumor.

The tumor will be rinsed to remove blood, the tissue will be ground, and *E. coli* will be added to amplify phage. Phage will be eluted from tumor cells and amplified using methods established by us and others [2, 5, 25, 61]. Phage will be amplified and quantified by titering, with results available within 12 hours. If (enriched) phage are present, they will be prepared as was

described above for initial infusion and will be reinjected as soon as possible (1 to 2 days). The harvest and amplification will be repeated a maximum of 2 times for a maximum of three screenings in one patient. Screening will ideally be completed in less than 7 days to avoid patient Ab response to phage [62]. Throughout the screening process patients will be carefully evaluated for adverse reactions.

Peptides displayed by phage isolated from tumor tissue will be routinely analyzed for both consensus amino acid sequences and tumor-binding as in Aim 3. Binding to normal tissue will be assessed by immunohistology on normal, quick frozen breast tissue excised at the same time as the tumor biopsy and on a large panel of 32 different normal human tissues not from the protocol patient. Immunohisotochemistry with anti-transferrin receptor mAb will be used as a positive control to assure tissue and assay reliability.

Any consensus sequences identified from phage eluted specifically from the tumor tissue will be excellent candidates for tumor-specific peptides. Promising peptides will be synthesized and tested for tumor specificity. Peptide binders we identify by whole body screens, almost by definition, are likely to be stable in serum and generally stable *in vivo*, a major advantage to this technique.

In future studies, we will evaluate the efficacy of peptide-therapeutic conjugates. In addition to attaching standard cytotoxic drugs to the ligands, in separate experiments we will attach immunogenic peptides, perhaps one to which most people have already been immunized against. The binding of a molecule bearing an immunogenic peptide to the tumor cell surface may stimulate the immune system to eliminate the tumor cell. Thus, instead of using dangerous chemicals or radiation, we may be able to direct the body's own immune system to more naturally eradicate tumor cells.

7.0 Pharmaceutical Information

Qualified personnel who are familiar with procedures that minimize undue exposure to themselves and to the environment will undertake the preparation, handling, and safe disposal of agents in a self-contained, protective environment.

Drug Information And Preparation:

Filamentous peptide-phage are prepared from *E. coli* cultures grown overnight on 2xYT media agar plates. The phage particles are resuspended in phosphate buffered saline with prokaryotic protease inhibitors (PBS-PPI) by "sweeping" the agar with an angled glass rod. The phage suspension is centrifuged twice to remove bacterial cells and filtered with a 0.22 um polyethersulfone membrane to completely remove any remaining *E. coli* cells. The phage are concentrated by precipitation with polyethylene glycol (PEG). The resulting pellet is resuspended in fresh PBS-PPI and the phage suspension is passed through a 0.45 μm cellulose acetate filter. Endotoxins are removed from the preparation by performing three 1% (v/v) Triton X-114 extractions. The phage are concentrated with PEG again and the resulting pellet is resuspended in PBS-PPI. The phage suspension is shaken 10 min at 200 rpm on ice, followed by centrifugation. The supernatant containing the peptide-phage is passed through a 0.45 μm cellulose acetate filter, followed by passage through a pyrogen-free 0.2 μm cellulose acetate filter to sterilize the preparation.

According to FDA guidelines, establishment of the sterility of any preparation to be injected into humans must be performed by inoculation of the product into Fluid Thioglycollate Media and Tryptic Soy Broth. We have performed these sterility tests exactly as described in the Code of Federal Regulations (21CFR610.12) on representative preparations. These tests have confirmed the sterility of our preparations, as expected after filtration through a pyrogen-free 0.2 μm cellulose acetate filter.

Although the sterilization filtration technique takes only a minute, the FDA guidelines for asserting sterility takes 14 days. This 14 day waiting period is not compatible with the screening experiments we have proposed for 2 reasons:

A) In our experience and judgement, when performing phage-display RPL screening, it is optimal to prepare a fresh batch of a phage-displayed library to optimize complexity of libraries, as some of the displayed peptides within the library may be more susceptible to degradation than other peptides. Ideally we would like to be able to establish proof of sterility, lack of endotoxins, and any other parameters necessary, within a few hours. It is possible that some of these difficulties may be avoided by freezing peptide-phage preparations. However, the freezing process may also compromise the stability of the peptides and therefore, the complexity of the libraries. It is possible that all the peptides which may bind to tumor targets may be stable for 14 days. This may be elucidated as the study progresses. Furthermore, freezing or other storage methods which would avoid peptide degradation for 14 days, will not address the problem raised in point B below. It may be possible to establish a large batch of libraries used for *initial* screenings only if a storage method which allows the displayed peptides to retain their tumor binding activity can be developed.

B) To perform 3 screens in one person, the screens will probably need to be performed in about 7 days, in order to avoid rejection of phage by the immune system, as a detectable IgM response to injected phage typically begins to develop in humans in about 7 days. Serial screening in one person may be the most optimal way to identify tumor-homing peptides, and may well be the only way to identify tumor-specific targets which are individual to a given patient. Therefore, to perform three screens in one patient, a 14 day waiting period for results of sterility testing will probably not be possible.

Administration: Peripheral intravenous line

8.0 Potential Toxicity, Dose Modifications, and Management

There is the risk of allergic reaction (including anaphylactic) associated with the administration of the phage library. The likelihood of this is very low since a similar (but different strain) of bacteriophage has been injected into thousands of patients with no serious sequelae. Personnel skilled in handling allergic reactions will be immediately present during infusions and all equipment for handling such reactions (including anaphylactic) will be immediately available. A small number of individual molecules of each type of displayed peptide are not expected to have strong toxicity. Preliminary studies in animals will help determine this. Patients will be closely monitored. All necessary measures will be taken to counter any level of allergic reaction.

All reagents will be prepared according to FDA standards and tested for sterility and pyrogenicity prior to administration. There is an extremely low risk of a patient receiving material that is either pyrogenic or not sterile.

Dr. Hans Ochs, a collaborator on this project, has the world's largest experience injecting bacteriophage and is a collaborator on this proposal. He has performed extensive studies with bacteriophage injected IV in humans. It has been used safely in over 3000 patients to monitor antibody responses with only rare adverse reactions in patients with unusual genetic immune deficiencies. Investigations were carried out to detect phage DNA incorporation into eukaryotic cells and was found only transiently in lymphocytes. He has an IND with the FDA for intravenous administration of bacteriophage. He also has extensive experience with the immunological consequences of intravenous administration of bacteriophage.

9.0 Criteria for Response Assessment

This is not a therapeutic trial; therefore the change in tumor size will not be assessed.

10.0 Removal of Patients from Protocol Therapy

If at any time the constraints of this protocol are detrimental to the patient's health and/or the patient no longer wishes to continue with the procedure, the patient shall be withdrawn from the study. In this event:

- Notify the study chair
- Document the reason(s) for withdrawal on flow sheets

11.0 Adverse Event Reporting

All adverse events (AEs) occurring with any patient participating in this clinical trial will be reported to the Cancer Center Protocol Office as described below.

11.1 Immediately (within 24 hours) telephone the Protocol Office for any of the following reasons:

- Any and all serious and/or life-threatening events which may possibly be reasonably associated, i.e., may reasonably be regarded as caused by, or reasonably be regarded as probably or possibly caused by, the investigational drug used in this protocol, or due to drug administration.
- All fatal and unexpected events regardless of cause or association with study treatment.
- All first occurrence of any "unexpected" (previously unobserved or unreported) toxicity (regardless of Grade).

11.2 Definitions

The following definitions of terms as per Federal Regulations apply to this section:

- *Serious adverse event*: any experience that is fatal or life threatening, is permanently disabling, requires inpatient hospitalization, or is a congenital anomaly, or overdose.

- *Associated with the use of the drug*: there is reasonable possibility that the experience may have been caused by the drug or combination of drugs.

- *Unexpected adverse event*: any adverse event that is not identified in nature, severity, or frequency in the current investigator's brochure or package insert.

11.3 Algorithm for Reporting Adverse Reactions

- Any and all serious and/or fatal or life threatening events which may be associated with the investigational drug used in this protocol, or due to drug administration. (see above):
- Report by telephone or facsimile transmission within 24 hours regarding to the Cancer Center Protocol Office at 802-656-2967.

Person to be contacted:

Paul Horton, Records Coordinator
2nd Floor Medical Alumni Building

Burlington, VT 05405
Phone: 802-656-2967
Fax: 802-656-8788
Email: phorton@zoo.uvm.edu

- Any and all serious and/or fatal or life threatening events which are not associated with the investigational drug used in this protocol or with drug administration should be reported to the Cancer Center Protocol Office **within 5 days.**

- A written report of all adverse effects or experiences and deaths will be submitted by the investigator/co-investigator. In this report, the investigator will advise whether or not the AE is judged to be attributable to the study medication. All such subjects should be followed clinically by the appropriate diagnostic studies. Side effect or subjective symptomatology volunteered by a subject will be noted and recorded as to type and severity on the individual's patient chart. If no side effects are experienced, this also will be reported on the patient chart.

- In turn the Protocol Office will inform the University Institutional Review Board (IRB) and the VCC Protocol Review Committee (PRC) and the FDA if necessary.

12.0 Statistical Considerations

Successful binding of ligand can be viewed as a dichotomous variable and this perspective will be taken relative to the examination of the data. Ligand binding will be examined at two distinct time points and the percent of subjects in whom a ligand is identified at each of the two time points will be initially quantified using exact 95% binomial confidence intervals. A sample size of n = 20 subjects would give rise to a standard error of at most 9.7% for a binomial point estimate under the assumption that the actual binding success rate would be at the 75% or higher level. With a 75% successful binding level, it is anticipated that an expected value of 7.5 out of the first 10 subjects examined would be observed. Since a change in the phage library would be desired if the library were not producing a sufficient rate of binding, we will examine the data in a sequential fashion. The probability of observing four or fewer successful events out of n = 10 subjects equals 2% using a cumulative binomial distribution with individual trial probability of success of 75%. Thus, if four or fewer successful events are observed in the first ten subjects, we will shift to a new phage library. If five or more of the first ten subjects have ligands identified, the next ten subjects will be examined using the same phage library. Since individual subject data are to be obtained at two distinct time points, the experimental design leads to a binary repeated measures setting. Each subject will be considered as a stratum within the context of a conditional logistic regression model. Given the small sample size, an exact procedure will be taken that assumes that the timing effects are conditionally independent of the stratum effects. The software package StatXact will be used to implement examination of the equality of the success rates levels at the two time points. Cross reactivity with other tissue types (total tissue types = 32) will be examined individually for each tissue type using contingency table methods with quantification of the level of cross reactivity using a 95% confidence interval for the odds ratios. Identification of the prevalence of common peptide motifs will be estimated using exact 95% binomial confidence intervals. It is anticipated that a 30% prevalence of a common motif will give rise to a point estimate with a standard error of about 9%.

13.0 Records to be Kept

Data on the tumor characteristics will be collected from the pathology reports, infusion data (amount, batch number) will be recorded, and the molecular consensus sequences of possible binding peptides will be documented.

14.0 References

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15.0 **Informed Consent**

INFORMED CONSENT

Protocol Title: In Vivo Selection of Ligands for Targeted Therapy

Protocol Chairman: David N. Krag, M.D.

Responsible Physician: _____

Sponsor: National Cancer Institute and Vermont Cancer Center, University of Vermont

I, _____, understand that I am being invited to participate in the above research study that has been explained to me by Dr. _____. It has been explained to me that I have breast cancer and have been invited to participate in a clinical study to identify new methods of selectively targeting and delivering anticancer drugs directly to a breast cancer.

Present treatments for breast cancer are often ineffective because they lack the ability to target cancer cells. Specifically, anticancer drugs are injected intravenously and distribute throughout the entire body. The drug itself has no particular preference for concentrating in cancer cells. Therefore the entire body is exposed to the same dose of toxic anticancer drugs. The effect of toxic anticancer drugs on normal non-cancer cells is a limiting factor. This means that the dose of drug given can only be so much. It may take considerably more drugs than the body can tolerate in order to cure a patient of breast cancer.

The ultimate goal of this clinical research is to develop a new way to target and selectively concentrate anticancer drugs to cancer cells. We propose to add a small tag, which is a molecule called a peptide, to the anticancer drug. This special tag would allow the anticancer drug to be delivered and concentrated at breast cancer cells. In this way, the concentration of drug would be much higher at the cancer cells than normal non-cancer cells.

The immediate goal of this protocol is to develop the tag that will attach to the "address" of cancer cells. The targeting agent (tag) will later be designed to deliver anticancer drugs directly to the address of the cancer cells. Right now we do not know the address of cancer cells. In order to find a tag to cancer cells we propose to intravenously inject several million different tags (or molecules). A small piece of the cancer is removed and the tags that have found their way to the cancer cells (out of the millions injected) and are able to stick at the address of cancer cells will be determined.

Attaching each of the tags to a special agent called a bacterial virus allows my doctor to find the special tag that sticks preferentially to the cancer cells. My doctor is able to find the bacterial viruses and is then able to determine the exact nature (or type) of the tag that sticks to my cancer.

The bacterial viruses (which carry the tag) are living agents that are similar to the human viruses (or germs) that cause the flu and other minor and sometimes serious illnesses in people. These bacterial viruses are special and are not known to infect humans or any other animals. They are not known to cause any human illnesses and may act to protect humans against bacterial infections. They are only able to infect a very small living cell called a bacterium. Many thousands of humans have been previously injected with special bacterial viruses (similar to the ones proposed here) and adverse responses are extremely rare.

Following injection of the special tags, my doctor will remove a small piece of cancer from my body. The size of this piece of cancer will be a cube about than 1/2 an inch on edge. The reason for removing this

piece is to find the special tags that have stuck to my cancer cells. This procedure will be a minor surgical procedure and may involve a small incision and placement of stitches to close the wound. This procedure will be done under local anesthesia. Several hours later another small piece of my cancer will be removed

It is expected that my doctor will find many tags in the cancer specimen that was removed from my body. Some of these tags are there because they truly stick strongly to my cancer cells. These are the tags that my doctor is after. Some of the tags, however, will be just passersby. That is, some of the tags will just happen to be passing though the cancer at the time the small specimen is removed and will not really be sticking to the cancer.

In order to determine which tags truly stick and which do not (or do not stick very well) my doctor will purify the tags found in my cancer and later reinject them into my vein. Just as was done following the first injection, a piece of cancer will be removed, and several hours later another small piece of my cancer will be removed. This time there will be many less types of special tags to bind to my cancer cells and it is more likely that my doctors will be able to identify the tags that stick most strongly to my cancer cells. The entire procedure of injection and biopsies will be repeated a third time. All of the injections and biopsies will be performed within 7 to 14 days.

Before the first injection takes place my doctor may perform a biopsy to have enough of my cancer cells available for later testing. The tags later identified to stick most strongly to my cancer cells will be tested against the first cancer biopsy material to determine how sticky they are.

This entire procedure: 1) biopsy cancer tissue, 2) injection of special tags in my veins and biopsy of cancer tissue, and 3) repeat injection of special tags and repeat biopsy of cancer tissue, is only one set of important steps required before this method of targeting anticancer drugs can be useful in possibly treating cancer. This clinical study is only designed to determine if this method (injection of tags and biopsy of cancer tissue) can find the special tags that stick preferentially to my cancer cells.

In order for this method to be possibly useful in treating my cancer an entire additional set of studies need to be performed. These additional studies involve connecting the special tag to anticancer drugs for special delivery to my cancer cells. It is important for me to understand that these second set of studies will not be performed as part of this clinical study I am being invited to participate in. That means that the findings from this clinical study will not necessarily be useful to me personally in treating my cancer.

BENEFITS AND RISKS:

I understand that I will be monitored very closely by either a nurse, or my doctor, or both, during the time of the injection of the material into my veins. I understand that there is a risk of allergic reaction to the material. Although the risk is very low (less than 1 in a thousand) it is real and may be serious and possibly life threatening. I understand that medications will be immediately available to counteract any allergic reactions, no matter how severe.

I understand that I will have an intravenous catheter placed into one of my veins. This is called an "IV" and it may be uncomfortable since it requires placement of a needle into my vein first. It will remain in my arm for several hours until the entire procedure is complete. I understand that a risk of having an intravenous catheter placed is an infection. This is a small risk but is real. If this should happen I may need to put warm soaks on the area and may need to take an antibiotic.

I understand that I will have up to seven biopsies of my cancer tissue. Each one of these biopsies will be small but will involve a small surgical procedure. I understand that local anesthesia will be injected around the biopsy site to numb the tissue. I may need to have stitches placed. There is a small risk of infection as

there is with any surgery. If this should happen I may need to put warm soaks on the area and may need to take an antibiotic.

I understand that this clinical study will be of no immediate or direct benefit to me. It may in the future lead to important findings that may benefit others. There is a remote possibility that if this study is successful I may participate in subsequent clinical studies designed to test the anticancer effectiveness of this technique.

ALTERNATIVE TREATMENTS:

I understand that my therapy will not be lessened by participation in this trial. I understand that this clinical study is not a therapeutic study and therefore not related to my treatments.

I have discussed the above facts with my physician and have been given the opportunity to ask questions which have been answered to my satisfaction. I understand that my physician will answer any questions that I might have.

COSTS AND PAYMENTS:

The injection of the special tagged material and the surgical biopsies will not be billed to me. Other medications and all physicians' and hospital costs related to my regular treatments will be charged to me in the same fashion as if I was not part of this study. I will receive no monetary compensation for my participation in this study.

CONFIDENTIALITY:

I understand that a record of my progress will be kept in a confidential form at the Vermont Cancer Center, University of Vermont (VCC/UVM). I understand that the results of this study may eventually be published and that information may be exchanged between medical investigators, but that patient confidentiality will be maintained. There is a possibility that my medical record, including identifying information, may be inspected and/or photocopied by qualified representatives from VCC/UVM, the National Cancer Institute or other Federal or state government agencies in the ordinary course of carrying out their governmental functions. If my record is used or disseminated for government purposes, it will be done under conditions that will protect my privacy to the fullest extent possible consistent with laws relating to public disclosure of information and the law-enforcement responsibilities of the agency.

RIGHT TO WITHDRAW:

I understand that I am free to refuse to participate in this trial or to withdraw at any time and that my decision will not adversely affect my care at this institution or cause a loss of benefits to which I might otherwise be entitled.

COMPENSATION FOR ILLNESS OR INJURY:

I understand that it is not the policy of the University of Vermont or Fletcher Allen Health Care to provide payment or free medical treatment for injury resulting from research. I understand that I may contact Dr. David Krag, One South Prospect St. (802-656-2262) for more information about this study or Nancy Stalnaker, the Institutional Review Board Administrator, 231 Rowell Building (802-656-4067) for information regarding my rights as a research subject or for information about how to proceed should I believe that I have been injured as a result of my participation in this study.

VOLUNTARY CONSENT:

I have been given an opportunity to discuss this trial with the physician(s) conducting it and I understand that I may ask further questions and that I may withdraw from the study at any time. Withdrawal from the study will not prejudice my further care at the Vermont Cancer Center or Fletcher Allen Health Care. I agree to participate in this study and I acknowledge that I have received a signed copy of this consent form.

_____ _____

Signature of Patient Date

_____ _____

Signature of Witness Date

_____ _____

Signature of Physician Date

Protocol Chairman:

David Krag, M.D.
Surgical Associates
One South Prospect
Burlington, VT, 05401
802-656-2262

APPENDIX I

NCI COMMON TOXICITY CRITERIA

TOXICITY (Adverse Event)	GRADE 0	GRADE 1 (Mild)	GRADE 2 (Moderate)	GRADE 3 (Severe)	GRADE 4 (or Life-Threatening)
BLOOD/BONE MARROW					
WBC	≥ 4.0	3.0 - 3.9	2.0 - 2.9	1.0 - 1.9	< 1.0
PLT	WNL	75.0 - normal	50.0 - 74.9	25.0 - 49.9	< 25.0
Hgb	WNL	10.0 - normal	8.0 - 10.0	6.5 - 7.9	< 6.5
Granulocytes/ Bands	≥ 2.0	1.5 - 1.9	1.0 - 1.4	0.5 - 0.9	< 0.5
Lymphocytes	≥ 2.0	1.5 - 1.9	1.0 - 1.4	0.5 - 0.9	< 0.5
LIVER					
Bilirubin	WNL	—	< 1.5 x N	1.5 - 3.0 x N	> 3.0 x N
Transaminase (SGOT, SGPT)	WNL	≤ 2.5 x N	2.6 - 5.0 x N	5.1 - 20.0 x N	> 20.0 x N
Alk Phos or 5' nucleotidase	WNL	≤ 2.5 x N	2.6 - 5.0 x N	5.1 - 20.0 x N	> 20.0 x N
GASTROINTESTINAL					
Nausea	none	able to eat reasonable intake	intake significantly decreased, but can eat	no significant intake	—
Vomiting	none	1 episode in 24 hours	2-5 episodes in 24 hours	6-10 episodes in 24 hours	> 10 episodes in 24 hours or requiring parenteral support
Diarrhea	none	increase of 2-3 stools/day over pre-Rx	increase of 4-6 stools/day, or nocturnal stools, or moderate cramping	increase of 7-9 stools/day, or incontinence, or severe cramping	increase of ≥10 stools/day, or grossly bloody diarrhea, or need for parenteral support
Stomatitis	none	painless ulcers, erythema, or mild soreness	painful erythema, edema, or ulcers, but can eat	painful erythema, edema, or ulcers, and cannot eat	requires parenteral or enteral support
KIDNEY/BLADDER					
Creatinine	WNL	< 1.5 x N	1.5 - 3.0 x N	3.1 - 6.0 x N	> 6.0 x N
Proteinuria	no change	1+ or <0.3 g% or < 3 g/l	2 - 3+ or 0.3 - 1.0 g% or 3 -10 g/l	4+ or > 1.0 g% or > 10 g/l	nephrotic syndrome
Hematuria	neg	micro only	gross, no clots	gross + clots	requires transfusion

APPENDIX I (Continued)

TOXICITY (Adverse Event)	GRADE 0	GRADE 1 (Mild)	GRADE 2 (Moderate)	GRADE 3 (Severe)	GRADE 4 (or Life-Threatening)
HEART/LUNGS					
Cardiac dysrhythmias	none	asymptomatic, transient, requiring no therapy	recurrent or persistent, no therapy required	requires treatment	requires monitoring; or hypotension, or ventricular tachycardia, or fibrillation
Cardiac function	none	asymptomatic, decline of resting ejection fraction by less than 20% of baseline value	asymptomatic, decline of resting ejection fraction by more than 20% of baseline value	mild CHF, responsive to therapy	severe or refractory CHF
Cardiac–ischemia	none	non-specific T-wave flattening	asymptomatic, ST and T wave changes suggesting ischemia	angina without evidence for infarction	acute myocardial infarction
Cardiac–pericardial	none	asymptomatic effusion, no intervention required	pericarditis (rub, chest pain, ECG changes)	symptomatic effusion; drainage required	tamponade; drainage urgently required
Pulmonary	none or no change	asymptomatic, with abnormality in PFTs	dyspnea on significant exertion	dyspnea at normal level of activity	dyspnea at rest
Weight gain/loss	< 5.0%	5.0 - 9.9%	10.0 - 19.9%	≥ 20.0%	—
BLOOD PRESSURE					
Hypertension	none or no change	asymptomatic, transient increase by greater than 20 mm Hg (D) or to > 150/100 if previously WNL; no treatment required	recurrent or persistent increase by greater than 20 mm Hg (D) or to > 150/100 if previously WNL; no treatment required	requires therapy	hypertensive crisis
Hypotension	none or no change	changes requiring no therapy (including transient orthostatic hypotension)	requires fluid replacement or other therapy	requires therapy and resolves within 48 hours of stopping the agent	requires therapy for > 48 hours after stopping the agent

APPENDIX I (Continued)

TOXICITY (Adverse Event)	GRADE 0	GRADE 1 (Mild)	GRADE 2 (Moderate)	GRADE 3 (Severe)	GRADE 4 (or Life Threatening)
NEUROLOGIC					
Neuro–sensory	none or no change	mild paresthesias, loss of deep tendon reflexes	mild or moderate objective sensory loss; moderate paresthesias	severe objective sensory loss or paresthesias that interfere with function	—
Neuro–motor	none or no change	subjective weakness; no objective findings	mild objective weakness without significant impairment of function	objective weakness with impairment of function	paralysis
Neuro–cortical	none	mild somnolence or agitation	moderate somnolence or agitation	severe somnolence, agitation, confusion, disorientation, or hallucinations	coma, seizures, toxic psychosis
Neuro–cerebellar	none	slight incoordination dysdiakokinesis	intention tremor, dysmetria, slurred speech, nystagmus	locomotor ataxia	cerebellar necrosis
Neuro–mood	no change	mild anxiety or depression	moderate anxiety or depression	severe anxiety or depression	suicidal ideation
Neuro–headache	none	mild	moderate or severe but transient	unrelenting and severe	—
Neuro–constipation	none or no change	mild	moderate	severe	ileus > 96 hours
Neuro–hearing	none or no change	asymptomatic, hearing loss on audiometry only	tinnitus	hearing loss interfering with function but correctable with hearing aid	deafness not correctable
Neuro–vision	none or no change	—	—	symptomatic subtotal loss of vision	blindness

APPENDIX I (Continued)

TOXICITY (Adverse Event)	GRADE 0	GRADE 1 (Mild)	GRADE 2 (Moderate)	GRADE 3 (Severe)	GRADE 4 (or Life Threatening)
DERMATOLOGIC					
Skin	none or no change	scatter macular or papular eruption or erythema that is asymptomatic	scatter macular or papular eruption or erythema with pruritus or other associated symptoms	generalized symptomatic macular, papular, or vesicular eruption	exfoliative dermatitis or ulcerating dermatitis
Palmar-Plantar Erythro-dysesthesia	no symptoms	Mild erythema, swelling, or desquamation not interfering with daily activities.	Erythema, desquamation, or swelling interfering with, but not precluding, normal physical activities; small blisters or ulcerations less than 2 cm in diam.	Blistering, ulceration, or swelling interfering with walking or normal daily activities; cannot wear regular clothing.	Diffuse or local process causing infectious complications, or a bed ridden state or hospitalization.
Allergy	none	transient rash, drug fever < 38°C, 100.4°F	urticaria, drug fever = 38°C, 100.4°F, mild bronchospasm	serum sickness, bronchospasm requiring parenteral medications	anaphylaxis
Alopecia	no loss	mild hair loss	pronounced or total hair loss	—	—
METABOLIC					
Hyperglycemia	< 116	116 - 160	161 - 250	251 - 500	> 500 or ketoacidosis
Hypoglycemia	> 64	55 - 64	40 - 54	30 -39	< 30
Amylase	WNL	< 1.5 x N	1.5 - 2.0 x N	2.1 - 5.0 x N	> 5.1 x N
Hypercalcemia	< 10.6	10.6 - 11.5	11.6 - 12.5	12.6 - 13.5	> 13.5
Hypocalcemia	> 8.4	8.4 - 7.8	7.7 - 7.0	6.9 - 6.1	≤ 6.0
Hypomagnesemia	> 1.4	1.4 - 1.2	1.1 - 0.9	0.8 - 0.6	≤ 0.5

APPENDIX I (Continued)

TOXICITY (Adverse Event)	GRADE 0	GRADE 1 (Mild)	GRADE 2 (Moderate)	GRADE 3 (Severe)	GRADE 4 (or Life Threatening)
COAGULATION					
Fibrinogen	WNL	0.99-0.75 x N	0.74-0.50 x N	0.49-0.25 x N	≤ 0.24 x N
Prothrombin time	WNL	1.01-1.25 x N	1.26-1.50 x N	1.51-2.00 x N	> 2.00 x N
Partial thrombo-plastin time	WNL	1.01-1.66 x N	1.67-2.33 x N	2.34-3.00 x N	> 3.00 x N
Hemorrhage (Clinical)	none	mild, no transfusion	gross, 1-2 units transfusion per episode	gross, 3-4 units transfusion per episode	massive, >4 units transfusion per episode

Source (modified from): National Institute of Health, National Cancer Institute, Cancer Therapy Evaluation Program, Bethesda, Maryland 20892

Chills (rigors)	none	any rigor, mild	rigors requiring medication	rigors not controlled by medication	—

Appendix II

DATA COLLECTION FORMS

Registration & Eligibility Checklist
VCC Confirmation of Registration
Adverse Event Report

In Vivo Selection of Ligands for Targeted Therapy
Patient Registration Form (Page 1)

PLEASE PRINT

Surgeon Of Record: _____

Contact Person: _____

Phone Number: _____ FAX : _____

Patient Name: _____

Birth Date: ____ / ____ / ____ Date of Diagnosis: ____ / ____ / ____

Signed Consent Attached? ____ Yes ____ No

FAX BOTH REGISTRATION PAGES AND THE ENTIRE CONSENT FORM TO:
802-656-1987

PATIENT WILL NOT UNDERGO PROCEDURE UNLESS THIS SHEET IS FAXED BACK TO YOU WITHASSIGNED ID NUMBER.

BELOW LINE FOR OPERATIONS CENTER USE ONLY

Assigned Patient ID Number: _____

Date of Registration: ____ / ____ / ____

CHRMS #: _____

Registrar: _____

Checklist:

Registration form legible and complete ___
Consent form legible and complete ___
Eligibility criteria are all checked "Y" ___
Consent form is correct for study ___
Consent form has not expired ___

VCC registration form submitted ____

In Vivo Selection of Ligands for Targeted Therapy

Patient Registration Form (Page 2)

Eligibility Criteria

Patient Name: _____ Date of Birth: _____

1. The patient has metastatic or locally advance primary or recurrent breast cancer that is not considered curable by conventional therapy. Y ___ N ___
2. The patient has superficial cancer nodules or mass amenable to biopsy with minor surgery. Y ___ N ___
3. The patient has a Karnofsky status ≥ 70 and a life expectancy ≥ 4 months. Y ___ N ___
4. The patient has undergone an informed consent process. Y ___ N ___
5. The patient is not pregnant. Y ___ N ___
6. The patient does not have any other serious illness, other than that treated by this study Y ___ N ___
7. The patient is 30 to 70 years of age. Y ___ N ___
8. The patient has no evidence of extensive pulmonary metastases. Y ___ N ___
9. The patient has no clinical symptoms suggestive of brain metastases. Y ___ N ___

All statements must be checked "Y" or "N/A" for entry into the trial.

Note: this sheet does not constitute source documentation. The above information must be included elsewhere in the patient's hospital chart in a recognized source document.

Signature of Physician: _____ Date: _____

VCC CONFIRMATION OF REGISTRATION

In Vivo Selection of Ligands for Targeted Therapy

Physician:_____

Patient Name:_____
(Please print) Last First Middle

Hospital Chart #:_____ Social Security # _____

Race: _____ Sex: Male Female Date of Birth ____/____/____
(1-White, 2-Hispanic, 3-Black, 4-Oriental,
5-Native Hawaiian, 6-Native American, 7-Indian,
8-Filipino, 9-Other, 10-Patient refusal, 1-Institution
refusal, -1-unkown)

Method of Payment_____ Zip Code:_____
(1-Private Insurance,2-Medicare, 3-Medicare
and private insurance, 4-Medicaid, 5-Medicaid and
Medicare, 6-Military or Veterans Administration
Sponsored, 7-Self Pay (no insurance), 8-No means of
payment (no insurance), -1-unknown)

Eligibility Criteria:- See protocol checklist
_____Patient Eligible? (1-no; 2-yes, all requirements confirmed)

If assignment is necessary from VCC, please fax to Donna Silver 802-656-8788 to get registration.

If different levels list here as 1,2 3.

Patient Study Number _____
Level Assigned _____
Date Registered_____
Registrar _____

VCC Registration Form

REPORT OF ADVERSE EVENTS AND/OR UNANTICIPATED PROBLEMS

All items on this form must be completed by the principal/co-investigator. Please attach any additional information.

Submit completed form to:

Cancer Center Protocol Office
2nd Floor Medical Alumni Building
Burlington, VT 05405

FOR VCC USE ONLY
Date of Notification
Initial Contact ___/___/___
FU Contact ___/___/___

CHRMS #: _____ PRINCIPAL INVESTIGATOR: David N. Krag, MD

PROTOCOL NUMBER AND TITLE: In Vivo Selection of Ligands for Targeted Therapy

Patient Identification Number: _____ Date of event/problem: _____

Brief description of event/problem (please do not indicate "see attached" as a response):

Did event/problem occur here? Yes _____ No _____

Was event/problem related to protocol? Yes _____ No _____ Unsure _____
If yes, how?

Have there been similar events/problems reported here? Yes _____ No _____
Elsewhere? Yes _____ No _____
If yes to either, explain:

Was the protocol discontinued for this subject? Yes _____ No _____ Unknown _____

Was further treatment required? Yes _____ No _____
If yes, explain:

Does the protocol need to be modified as a result of this report? Yes _____ No _____
If yes, explain:

Does the consent form need to be modified as a result of this report? Yes _____ No _____
If yes, explain:

Note: You should keep a copy of this completed form as this information *must* be included in your summary of events/problems encountered during the indicated time period of *your next continuing* review.

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In Vivo Selection of Ligands for Targeted Therapy

PRINCIPAL INVESTIGATOR
David N. Krag, MD
309C Given Building
University of Vermont
Tel: 656-5830
Fax: 656-5833
Email: dkrag@salus.med.uvm.edu

OPERATIONS CENTER
Vermont Cancer Center
24C Hills Science Building
University of Vermont
Tel: 656-4270
Fax: 656-1987

TABLE OF CONTENTS

1.0 INTRODUCTION

A major problem in the treatment of melanoma is that present therapies lack specificity for tumor cells and are extremely toxic to normal cells. The development of therapies with high specificity for tumor is an utmost priority in melanoma research.

Our lab is attempting to identify novel *small* ligands (1-2 kD) that bind specifically to tumor cells. Small ligands may have therapeutic activity alone, presumably by inhibiting a target molecule that actively plays a role in carcinogenesis. Small ligands can also be coupled to cytotoxic agents and used to mediate the specific destruction of tumor cells, even if their target molecules do not play an active role in cancer progression, as long as they are specifically present on the tumor (or on blood vessels specifically supplying tumor.) Ligands much smaller than antibody fragments may have important advantages in targeted therapy including improved tumor to non-tumor uptake ratios, better penetration of solid tumors, and non-immunogenicity. Small molecules are also easier to synthesize in the large amounts necessary for clinical use, are less likely to interfere with the effects of conjugated cytotoxic drugs, and may have improved specificity as there is less surface to interact non-specifically with other body components.

Large libraries of small compounds are a rich source of small ligands that may target tumors. Several types of these libraries, which consist of millions or even billions of different peptides, oligonucleotides, or synthetic molecules, have been constructed and used to isolate small ligands or lead compounds to many targets. The construction of libraries like these and their use in the identification of specific ligands, known as combinatorial technology, has revolutionized the field of drug discovery [28]. This proposal describes the use of this technology to identify small *peptide* ligands that will specifically bind to tumor cells and not to normal cells.

Although peptides have traditionally been discounted as potential therapeutics due to an assumption of their instability *in vivo*, peptides can form an almost infinite number of shapes and are exactly what nature uses to specifically target molecules both intracellularly and extracellularly. Many peptides have important biologic functions and potent *in vivo* activities. Furthermore, the exciting work of Ruoslahti et al [2, 60, 61, 65] has demonstrated that many peptides are stabile enough in serum to home specifically to tumors and to various organs. Elegant experiments from Affymax [18, 90] also demonstrated that, with minimal modification, peptides are capable of strong binding and effective agonist activity *in vivo* to cell surface receptors. Furthermore, small peptide ligands which are identified to tumor targets by *in vitro* screening, even if lacking *in vivo* stability, can be modified and/or used as a prototype in order to develop a small molecule which will be more stable and effective *in vivo*. We have already identified such a small peptide ligand to a potential tumor target, Grb2, and our collaborators have modified it chemically such that it retains binding activity in cell lysates [59]. Cyclic peptides are more stable *in vivo* and are often more selective for their targets. Many peptide libraries, including those used in our lab, are biased for cyclic peptides. D-amino acids, non-natural amino acids, and pseudo-peptide bonds may also confer greater *in vivo* stability. A novel and intriguing method of obtaining mimetics that may be more stable *in vivo* involves using a D-amino acid synthesized target. Screening with the D-amino acid target can result in the identification of D-amino acid peptide ligands to natural L-amino acid targets [71]. Peptide structures can also be readily determined by NMR and used to model peptidomimetics that may be more stable *in vivo*. The structure of peptide ligands can also provide important information about the structure of both their receptor targets and the natural ligands of those targets. Identification of peptide ligands can also facilitate the discovery of natural ligands to orphan receptors. Other advantages of peptide ligands for tumor targeting are that they can be easily synthesized in the large amounts needed for clinical use, their chemistry is well known, and conjugation methods are routine.

Peptides and peptidomimetics are very promising targeting agents because they can potentially bind targets with the same exquisite specificity as antibodies, and are likely to have

far more favorable pharmacokinetics. Peptides can have direct agonist or inhibitory activity on therapeutic targets. In another project, our lab is attempting to identify peptides that directly inhibit the dimerization of ErbB2, an effect that may result in therapeutic activity. Alternatively, peptide motifs can be used to direct other agents that have therapeutic activity, such as cytotoxic drugs, immune modulating agents, ribozymes, and gene therapy delivery systems such as liposomes or viral particles, to a specific molecular target. In a remarkable recent report, a short peptide sequence was grafted onto a 41kD protein capable of inhibiting growth factor receptor signaling [69]. The peptide allowed the protein to penetrate the cell membrane and to be delivered into the cytosol from the extracellular environment without detectable proteolysis. The delivered protein was successful in inhibiting growth factor signal transduction. Peptides can also potentially target imaging agents for diagnostic purposes.

Using a combinatorial approach, large random peptide libraries (RPLs) have been constructed in several systems. The RPLs described in this proposal have been or will be constructed in a phage-display system [19, 21, 83]. Phage-displayed and other biological RPLs are particularly powerful in that the peptides are physically linked to their encoding DNA. Because DNA is easily amplified for sequencing, one binding peptide out of millions can be determined. Phage-displayed libraries are made using filamentous phage that infect and multiply in *E. coli*. Each phage particle has five copies of a minor coat protein (pIII) located at one end. Random synthetic DNA is inserted into the gene coding for pIII so that the foreign DNA is expressed at the free N-terminus of pIII as random peptides. In this system, up to 5 copies of each peptide are physically "displayed" by each phage particle. (Another commonly used phage-display system employs the major coat protein and displays several hundred peptides per particle.) Each phage particle displays a different peptide. A phage particle bearing a peptide which bonds to a target can be isolated using affinity selection and is easily amplified in *E. coli*. After amplification the phage DNA can be sequenced to deduce the identity of the displayed peptide. The small size of the library particles allows manipulation of millions of different potential binding units in a few microliters.

Phage-displayed RPLs have been used by our lab and others to isolate small ligands, some with nanomolar and even picomolar affinity, to a large variety of targets including several potential tumor targets and other clinically important targets [2, 18, 60, 61, 65, 90]. One example of the use of small peptides (8 and 12 mer) in targeting tumors has been reported by Renschler et al. [66, 67] who used phage displayed RPLs to identify peptides that bind to the antigen binding receptor of B-lymphoma cells and induce apoptosis *in vitro*. Most of these ligands have been identified using *in vitro* screening techniques: binding purified target protein to a matrix, incubation of the immobilized protein with the peptide-phage library, washing away non-specific binders, elution of specifically bound phage, followed by phage amplification and DNA sequencing to determine the identity of the peptide responsible for binding activity.

This proposal describes the use of phage-displayed RPL technology to identify small peptide ligands to melanoma-specific targets by *in vivo* screening in melanoma patients. In future studies, these peptides will be modified if necessary to optimize *in vivo* stability, coupled to cytotoxic or other therapeutic agents, and used to mediate the specific destruction of melanoma tumor cells. Proof of concept for our project is found in several exciting recent reports [2, 18, 60, 61, 65, 90], one which describes *in vivo* screening of RPLs similar to ours in mice bearing human tumor xenografts and the identification of peptides which home specifically to tumor blood vessels. Administration of peptide-doxorubicin conjugates to tumor-bearing mice resulted in a marked decrease in doxorubicin toxicity, selective tumor destruction, and excellent animal survival [2, 4]. The same group has also identified peptides that bind preferentially *in vivo* to at least 10 different organs [61, 65], further demonstrating the powerful ability of small peptides to home to specific molecular "addresses" in the body. These important findings have introduced a whole new field of exploration in the search for more specific and effective cancer therapeutics.

We are in a unique position to perform the novel *in vivo* RPL human screening experiments described in this proposal. We have extensive experience with phage RPLs and

have successfully developed binders to several potentially clinically useful targets. The PI has extensive clinical experience in the IV injection of experimental compounds such as radiolabeled monoclonal antibodies and the intratumor injection of technetium colloid materials. In vivo RPL screening experiments [2,4,61,65] are certainly among the most intriguing and novel in cancer research today. We feel it is important and urgent to pioneer similar techniques in humans, as the identification of ligands that recognize mouse endothelial cells may not be relevant to treatment of human melanoma.

In vivo RPL screens in humans offer several potentially critical advantages over *in vitro* screening including:

1) Tumor targets will be in their native conformation with all their human post-translational modifications. Screening targets in their native *in vivo* conformation may be especially important in light of the following: host endothelial targets may be among the most promising "tumor" targets available [10-12, 27, 37, 60] [4] and "the phenotype of endothelial cells is unstable and likely to change when the cells are removed from their microenvironment [3, 8, 65]." Therefore, IV injection of RPLs *in vivo* may not only be the *optimal* method of presenting these important endothelial targets to the library- it may be the *only* effective method. Host targets, which are genetically stable and homogenously present on target tissues, are particularly attractive due to the heterogenous nature of most cells of a tumor, which may be difficult to target with even a combination of drugs. However, targets located directly on tumor cells are also most advantageously presented in their native conformation.
2) Inherent selection of peptides that recognize *specific* targets due to efficient "subtraction" of library clones which bind to normal tissue during exposure of the injected library to the entire body. Efficient removal of library clones that bind normal tissue is essential to the recovery of tumor-specific clones. It would be difficult to "subtract" all clones that bind to every possible normal tissue by *in vitro* subtraction methods, as this would require having fresh tissue samples from dozens of human tissues harvested from a cadaver. Subtraction with fixed tissues may not give satisfactory results since all targets do not retain native conformation during tissue fixation. Obviously, *in vitro* subtraction with all possible normal tissues, fresh or fixed, would be impossible to do with the normal tissue of the same patient being screened. Rajotte *et al.* [65] noted how their *in vivo* screening method "surprisingly... consistently yielded tissue-specific homing peptides" and attribute this success to efficient whole body "elimination of nonspecific phage." Further evidence that *in vivo* screening methods may be more effective than *in vitro* methods in the selection of specific ligands to cell surface targets is supplied in an interesting report by Barry et al [5]. Using whole cells to pan *in vitro*, peptides were identified which bound to many cell types rather than peptides that bound specifically to the cell type used for screening.
3) Inherent selection of *only* peptides that are stable *in vivo*.
4) Inherent targeting of *only* targets that are stable *in vivo* and which are capable of stably binding ligands *in vivo*.
5) Purification or prior knowledge of particular targets is not necessary.
6) Potential elucidation of novel tumor targets. Targets are not influenced by immunogenicity as with targets defined only by monoclonal antibody development [5].

Screening whole cells or cell extracts in vitro can also achieve the latter two advantages; however, many of the important advantages will be lost using this technique. Similarly, while direct injection of tumor with RPLs for screening is certainly possible, the important advantage of whole body elimination of normal tissue binding clones will be lost, and the chance of exposing the library to the important apical surface of endothelial cells will be greatly decreased. Systemic injection may well be the most effective way to identify ligands

with a good tumor/control tissue- binding ratio. For example, in previous in vivo screening experiments, a peptide with a RGD motif binds integrins three orders of magnitude better than an NGR peptide. However, the NGR peptide had a tumor/control tissue homing ratio three times better than that of the RGD peptide [2].

The following proposal will extend *in vivo* screening techniques in the following ways to optimize its success in identifying small tumor-specific ligands in humans:

1) Identification of *human* targets. Ligands to mouse endothelial targets are not likely to be as effective or effective at all for human targets. In the previous *in vivo* tumor screening study in mice, many of the tumor-homing peptides contained an RGD amino acid motif, as did one of the two peptides assessed for their ability to direct doxorubicin to tumors [2]. Although integrins and related receptors are very promising tumor targets, and many integrin binding sequences contain an RGD motif, the regions flanking RGD are critical to the specificity of integrin binding. Optimal flanking regions of these integrin binding peptides are not likely to be identical for the recognition of both mouse and human integrins on newly developed endothelial cells, as mouse and human integrins are not identical as assessed by GCG analysis. Peptides identified by <u>human</u> *in vivo* screenings are far more likely to yield specific and high affinity binders to <u>human</u> tumors.
2) Identification of targets in a realistic setting. The mouse model was a xenograft model, and did not involve mouse tumor. This artificial model may yield results not applicable to the eradication of natural tumors.
3) By using a large panel of libraries that offer a variety of structural contexts for peptide presentation, we believe we may isolate peptides that bind to other promising melanoma targets as well as to tumor-associated integrins. It is likely that a successful therapeutic regimen for advanced cancer will require a "cocktail" of anti-cancer compounds. Targeting a single cancer target is not likely to effect a long-term favorable therapeutic response. In addition, there is evidence that other sequences may bind as tightly and more specifically than RGD sequences to integrins [9]. It is also possible that RGD and NGR peptides [2] may bind to too many targets to be specific enough. Even low affinity binding can have significant biological consequences, particularly with avidity effects, as is the case with integrin binding to fibronectin. Furthermore, a large panel of libraries which present a vast number of peptides presented in a variety of structural contexts is much more likely to yield a high affinity binder to any given target [7]. We will also employ several innovative RPL technical improvements, developed by both our lab and Affymax.
4) We will attempt to identify peptide ligands to endothelial targets by harvesting tissue within 10 minutes after library injection. However, by harvesting tissue 24 hours after library injection, we will also attempt to identify peptides that bind directly to tumor cells or are taken up by tumor cells.
5) We will attempt to assay peptide binding directly rather than via competition with peptide-phage as done in previous *in vivo* screening experiments. This will allow us to more accurately determine the degree of specificity by immunohistochemical analysis of peptides on tumor tissues and a large panel of normal tissues.
6) By performing multiple screens in one person, rather than serially as was done with mice, we may determine which targets are *unique* to a particular individual. (Important *generic* targets will be identified by similarities in consensus sequences identified from different patients in whom complete multiple screenings were performed, or by serial screens between different patients.) Even though screens will be initially performed on patients with advanced disease, it is very conceivable to establish a profile of ligands against the majority of newly diagnosed patients with melanoma immediately prior to

definitive surgery. This would allow design of systemic adjuvant therapy most appropriate to each patient.

7) Very importantly, we will carefully investigate toxicity during phage injections and screenings in both animals and humans. Toxicity during *in vivo* RPL screening has not previously been studied.
8) We will test several screening designs, including multiple screenings in one patient to determine the safest and most effective screening protocol.

A large portion of the following proposal is designed to examine possible toxicity during *in vivo* screening. We do not expect the screening procedure to cause toxicity as bacteriophage have been injected intravenously in humans and even neonates for over 30 years in approximately 3000 patients with essentially no side effects. This is extensively reported in the literature [13, 36, 58, 62, 85] (also see Appendix.) Phage are injected into humans IV routinely for analysis of antibody responses.

There has also been extensive use of over 250 strains of bacteriophage, including 39 that infect *Escherichia* bacteria, which were administered orally or locally for treatment of infection [76-82]. Not only were "side effects" described as "extremely rare" (3 allergic responses out of 138, with no prior endotoxin testing), the phage treatments were often effective in eliminating the bacterial infection.

Bacteriophage are known to specifically infect only bacteria, and each bacteriophage strain infects only a very narrow range of bacterial species. In addition, it has long been known that ssDNA is expressed poorly or not at all in mammalian cells. The presence of phage DNA in human cells was examined in the antibody analysis studies mentioned above several months after injection and none was detected. Therefore the possibility of bacteriophage "infecting" eukaryotic cells in any way is highly unlikely. Nevertheless, we will carefully monitor this possibility by PCR.

Dr. Hans Ochs, an immunologist who is one of the pioneers of using phage ϕX174 for antibody response analysis, is an enthusiastic collaborator on this project. Dr. Och's group has had an IND from the FDA for over 20 years for this procedure. We will have his expertise available to us for consultations on human phage injection and he will provide us with his strain of phage for library construction if necessary. Not only has the work of Och's et al been extensively documented for non-toxicity, their experiments give us an accurate estimate of the time we have available to screen before we expect immune system interference. Our library is constructed in a strain of bacteriophage different than the strain used for antibody response analysis, although they both infect *E. coli* exclusively. However, filamentous phage injection is not likely to cause toxicity, as there have been numerous reports of the injection of filamentous phage into mice for several other purposes including hybridoma development. There are several groups investigating the use of filamentous phage particles as potential vaccine delivery agents, with numerous preliminary studies in mice. At least one of these studies reports IV injection of a very large number ($2x10^{12}$) of phage particles with no toxicity reported, although toxicity was not explicitly addressed [32]. The mice survived at least 3 to 4 days when they were sacrificed in order to harvest spleen cells for hybridoma development. Our phage will also bear peptides, which may present complications particularly after amplification from tumor tissue, which will likely have normal tissue components. Therefore, the proposal has been carefully designed to detect any possible toxicity. We have had an active dialogue going on for several months with both the NCI and the FDA on this matter, and their input has shaped the present design.

2.0 OBJECTIVES

Specific Aim: T*he safety of IV administration of phage RPLs in human patients with melanoma will be established with both naïve libraries and amplified libraries.* These studies will be the equivalent of Phase I trials. Screenings will be performed three times over a time period of <14

days. Throughout the screening process patients will be carefully evaluated for adverse reactions. Peptides displayed by phage isolated from tumor tissue will be routinely analyzed for both consensus amino acid sequences and tumor-binding. Binding to normal tissue will be assessed by immunohistology on a large panel of 35 different normal human tissues. *Successful completion of this aim will establish the safety of in vivo phage RPL screening in humans and will result in the identification of peptides which bind specifically to melanoma tumor cells or to blood vessels specifically supplying tumor cells in human patients. Whole body in vivo screening experiments will result in the development of methods that may allow identification of novel tumor targets and greatly improved therapeutics.*

Hypotheses 1. Small peptides can be identified from RPLs that will bind specifically to human tumor cells and/or to blood vessels specifically supplying tumor by *in vivo* screening in human melanoma patients. 2. One way of identifying these peptides, and perhaps the most efficient way, is to inject phage RPLs libraries into patients and to harvest specific peptide-phage directly from resected tumor tissue. 3. Small ligands that bind specifically to cancer cells will allow development of more effective cancer therapeutics.

3.0 PATIENT ELIGIBILITY CRITERIA

3.1 **Histologic documentation:** patients with metastatic or locally advanced primary or recurrent melanoma that is not considered curable by conventional therapy.

3.2 **Prior Treatment:** No Limitations.

3.3 **Measurable Disease:**
Superficial cancer nodules or mass amenable to biopsy with minor surgery.

3.4 Age limitations: 30 – 70 years of age
Performance score: Karnofsky status ≥ 70
Life expectancy: ≥ 4 months
Non-pregnant

3.5 **Informed Consent:** the patient must be aware of the nature of his/her disease and willingly consent after being informed of the procedure to be followed, the experimental nature of the procedure, alternatives, potential benefits, side effects, risks, and discomforts.

3.6 **No previous or concurrent malignancy** is allowed, except inactive non-melanoma skin cancer, in situ carcinoma of the cervix, or other cancer if the patient has been disease-free for ≥ 5 years.

3.7 **No other serious medical illness,** other than that treated by this study, which would limit survival to < 4 months, or psychiatric condition which would prevent informed consent.

3.8 **No evidence of extensive pulmonary metastases** evidenced by chest roentgenogram within 60 days of the protocol.

3.9 **No clinical symptoms suggestive of brain metastases** unless ruled out by imaging studies.

4.0 Patient Registration

4.1 Authorized physicians or designees must fill out confirmation of registration sheet (Appendix II) fax into the Operations Center 656-4270 to obtain a patient number. Office hours are 8:00 to 4:30.

4.2 All patients will sign an approved informed consent that provides full disclosure of the procedure, rationale, plan, and risks.

4.3 At the time of registration, the investigator may be asked to respond to a list of questions related to the patient's eligibility for this protocol. The eligibility checklist is located in Appendix II

4.4 When a patient is removed from protocol (e.g., because of disease progression or drug-related toxicity), the Operations Center is to be called and given the reason for the patient's removal from the protocol and the date on which discontinuation of protocol occurred.

4.5 We estimate that we will study 20 patients.

5.0 Required Monitoring of Patients

Patients will be admitted to the General Clinical Research Center (GCRC). An intravenous line will be placed. Baseline vital signs (blood pressure, pulse, temperature, and respiratory rate) will be determined before infusion of phage, every 15 minutes during infusion and for 2 hours after infusion. The patient will be under constant supervision and equipment, medications, and personnel capable of treating allergic (including anaphylactic reactions) will be immediately available.

	Before Infusion	Every 15 Minutes During Infusion	Two Hours after Infusion
Blood Pressure	X	X	X
Pulse	X	X	X
Temperature	X	X	X
Respiratory Rate	X	X	X

Screening will ideally be completed in less than 7 days to avoid patient Ab response to phage [62]. Throughout the screening process patients will be carefully evaluated for adverse reactions.

6.0 Study Plan

Approximately 20 patients will be subjected in vivo screening in an attempt to identify peptides that home specifically to their tumor tissue. It is highly unlikely that cancer patients have preexisting intact filamentous phage located in their tumor tissue. Nevertheless, as a control, biopsies from two patients, uninjected with phage, will be assayed for the presence of phage. In the experimental group, if phage clones isolated from tumor tissue after the third tissue harvest display a consensus amino acid sequence, it is highly likely that those peptide-phage are binding specifically to some component of the patient's tissue. An internal control will be to compare the sequences of phage clones eluted from the first tissue harvested (expected to be relatively random) compared to the last tissue harvested, as well as comparing clone sequences from peptide-phage isolated from different patients. Regarding the ability of this procedure to identify peptides which home specifically to tumors, we do not believe methods to minimize bias on the part of subjects, investigators, and analysis are necessary, as the measurements to be made: the number of phage eluted from the tumor, and the sequence of the peptides displayed

by phage clones eluted from tumor, are objective and not subject to human bias. Regarding the possible side effects caused by this procedure, bias will be minimized by having all procedures performed in the University of Vermont General Clinical Research Center (GCRC). The staff at the GCRC are expert in observation of patients during experimental protocols and are not supervised by the PI of this investigation.

Patients will be admitted to the GCRC. An intravenous line will be placed. Baseline vital signs (blood pressure, pulse, temperature, and respiratory rate) will be determined before infusion of phage and every 15 minutes during infusion and for 2 hours after infusion. The patient will be under constant supervision and equipment, medications, and personnel capable of treating allergic (including anaphylactic reactions) will be immediately available. Anti-bacteriophage antibodies will be determined by ELISA prior to administration of the bacteriophage library. Based on previous studies [13, 36, 58, 62] very few individuals have pre-existing bacteriophage antibodies and it is expected that antibacteriophage antibodies will develop subsequent to phage library administration similar to that demonstrated for ϕX174 (see Ochs IND # BB-IND 714). Since positive titers are expected and serial administration of bacteriophage has resulted in only rare reversible side effects, subsequent titer levels will not be obtained for this protocol. In the event that the number of phage recovered from the tumor specimens harvested after the second phage library administration are extremely low, titer levels of anti-phage antibodies will be determined in order to determine if antibodies are the cause of low phage recovery.

Ideally, we will screen our RPL(s) three times in the same patient. In the unlikely event of toxicity with naïve libraries, no further pans will be performed. In the event that only amplified phage show evidence of toxicity, no further pans will be performed.

A phage displayed RPL pool will be prepared and tested according to FDA standards as discussed below in "Pharmaceutical Information/Preparation". Before phage injection, one sample of tumor tissue from the patient will be biopsied, snap frozen and sixty slides will be prepared for later testing of selected clones for tumor binding affinity. The library will be diluted in 250 ml saline and infused intravenously over 10 minutes into a melanoma patient. Initially $10^{9\text{-}10}$ pfu will be injected as that amount was found to be completely non-toxic to humans in similar studies [62]. Higher numbers of phage, up to $10^{14\text{-}16}$ or more may be necessary if we fail to harvest phage from a small amount of resected tumor. An appropriate dose will be one in which peptide-phage are isolated from small tumor biopsies, and preferably, one in which peptide-phage isolated from tumor tissue display homologous amino acid sequences. Small amounts of tumor tissue will be biopsied in the procedure room of the GCRC at time points of 10 minutes and 24 hours post infusion. Standard surgical technique typical for performing a surgical biopsy will be utilized for the biopsy procedures. It is intended that the biopsies be as small as possible on the order of 1.0 gram of tissue. The method of biopsy will be either incisional, excisional, or core depending on the location of the tumor.

The tumor will be rinsed to remove blood, the tissue will be ground, and *E. coli* will be added to amplify phage. Phage will be eluted from tumor cells and amplified using methods established by us and others [2, 5, 25, 61]. Phage will be amplified and quantified by titering, with results available within 12 hours. If (enriched) phage are present, they will be prepared as was described above for initial infusion and will be reinjected as soon as possible (1 to 2 days). The harvest and amplification will be repeated a maximum of 2 times for a maximum of three screenings in one patient. Screening will ideally be completed in less than 7 days to avoid patient Ab response to phage [62]. Throughout the screening process patients will be carefully evaluated for adverse reactions.

Peptides displayed by phage isolated from tumor tissue will be routinely analyzed for both consensus amino acid sequences and tumor-binding as in Aim 3. Binding to normal tissue will be assessed by immunohistology on normal, quick frozen melanoma tissue excised at the same time as the tumor biopsy and on a large panel of 32 different normal human tissues not from the protocol patient. Immunohisotochemistry with anti-transferrin receptor mAb will be used as a positive control to assure tissue and assay reliability.

Any consensus sequences identified from phage eluted specifically from the tumor tissue will be excellent candidates for tumor-specific peptides. Promising peptides will be synthesized and tested for tumor specificity. Peptide binders we identify by whole body screens, almost by definition, are likely to be stable in serum and generally stable *in vivo*, a major advantage to this technique.

In future studies, we will evaluate the efficacy of peptide-therapeutic conjugates. In addition to attaching standard cytotoxic drugs to the ligands, in separate experiments we will attach immunogenic peptides, perhaps one to which most people have already been immunized against. The binding of a molecule bearing an immunogenic peptide to the tumor cell surface may stimulate the immune system to eliminate the tumor cell. Thus, instead of using dangerous chemicals or radiation, we may be able to direct the body's own immune system to more naturally eradicate tumor cells.

7.0 Pharmaceutical Information

Qualified personnel who are familiar with procedures that minimize undue exposure to themselves and to the environment will undertake the preparation, handling, and safe disposal of agents in a self-contained, protective environment.

Drug Information And Preparation:
Filamentous peptide-phage are prepared from *E. coli* cultures grown overnight on 2xYT media agar plates. The phage particles are resuspended in phosphate buffered saline with prokaryotic protease inhibitors (PBS-PPI) by "sweeping" the agar with an angled glass rod. The phage suspension is centrifuged twice to remove bacterial cells and filtered with a 0.22 um polyethersulfone membrane to completely remove any remaining *E. coli* cells. The phage are concentrated by precipitation with polyethylene glycol (PEG). The resulting pellet is resuspended in fresh PBS-PPI and the phage suspension is passed through a 0.45 μm cellulose acetate filter. Endotoxins are removed from the preparation by performing three 1% (v/v) Triton X-114 extractions. The phage are concentrated with PEG again and the resulting pellet is resuspended in PBS-PPI. The phage suspension is shaken 10 min at 200 rpm on ice, followed by centrifugation. The supernatant containing the peptide-phage is passed through a 0.45 μm cellulose acetate filter, followed by passage through a pyrogen-free 0.2 μm cellulose acetate filter to sterilize the preparation.

According to FDA guidelines, establishment of the sterility of any preparation to be injected into humans must be performed by inoculation of the product into Fluid Thioglycollate Media and Tryptic Soy Broth. We have performed these sterility tests exactly as described in the Code of Federal Regulations (21CFR610.12) on representative preparations. These tests have confirmed the sterility of our preparations, as expected after filtration through a pyrogen-free 0.2 μm cellulose acetate filter.

Although the sterilization filtration technique takes only a minute, the FDA guidelines for asserting sterility takes 14 days. This 14 day waiting period is not compatible with the screening experiments we have proposed for 2 reasons:

A) In our experience and judgement, when performing phage-display RPL screening, it is optimal to prepare a fresh batch of a phage-displayed library to optimize complexity of libraries, as some of the displayed peptides within the library may be more susceptible to degradation than other peptides. Ideally we would like to be able to establish proof of sterility, lack of endotoxins, and any other parameters necessary, within a few hours. It is possible that some of these difficulties may be avoided by freezing peptide-phage preparations. However, the freezing process may also compromise the stability of the peptides and therefore, the complexity of the libraries. It is possible that all the peptides which may bind to tumor targets may be stable for 14 days. This may be elucidated as the study progresses. Furthermore, freezing or other storage methods which would avoid peptide degradation for 14 days, will not address the problem raised in point B below.
It may be possible to establish a large batch of libraries used for *initial* screenings only if a storage method which allows the displayed peptides to retain their tumor binding activity can be developed.
B) To perform 3 screens in one person, the screens will probably need to be performed in about 7 days, in order to avoid rejection of phage by the immune system, as a detectable IgM response to injected phage typically begins to develop in humans in about 7 days. Serial screening in one person may be the most optimal way to identify tumor-homing peptides, and may well be the only way to identify tumor-specific targets which are individual to a given patient. Therefore, to perform three screens in one patient, a 14 day waiting period for results of sterility testing will probably not be possible.

Administration: **Peripheral** intravenous line

8.0 Potential Toxicity, Dose Modifications, and Management

There is the risk of allergic reaction (including anaphylactic) associated with the administration of the phage library. The likelihood of this is very low since a similar (but different strain) of bacteriophage has been injected into thousands of patients with no serious sequelae. Personnel skilled in handling allergic reactions will be immediately present during infusions and all equipment for handling such reactions (including anaphylactic) will be immediately available. A small number of individual molecules of each type of displayed peptide are not expected to have strong toxicity. Preliminary studies in animals will help determine this. Patients will be closely monitored. All necessary measures will be taken to counter any level of allergic reaction.

All reagents will be prepared according to FDA standards and tested for sterility and pyrogenicity prior to administration. There is an extremely low risk of a patient receiving material that is either pyrogenic or not sterile.

Dr. Hans Ochs, a collaborator on this project, has the world's largest experience injecting bacteriophage and is a collaborator on this proposal. He has performed extensive studies with bacteriophage injected IV in humans. It has been used safely in over 3000 patients to monitor antibody responses with only rare adverse reactions in patients with unusual genetic immune deficiencies. Investigations were carried out to detect phage DNA incorporation into eukaryotic cells and was found only transiently in lymphocytes. He has an IND with the FDA for intravenous administration of bacteriophage. He also has extensive experience with the immunological consequences of intravenous administration of bacteriophage.

9.0 Criteria for Response Assessment

This is not a therapeutic trial; therefore the change in tumor size will not be assessed.

10.0 Removal of Patients from Protocol Therapy

If at any time the constraints of this protocol are detrimental to the patient's health and/or the patient no longer wishes to continue with the procedure, the patient shall be withdrawn from the study. In this event:

- Notify the study chair
- Document the reason(s) for withdrawal on flow sheets

11.0 Adverse Event Reporting

All adverse events (AEs) occurring with any patient participating in this clinical trial will be reported to the Cancer Center Protocol Office as described below.

11.1 Immediately (within 24 hours) telephone the Protocol Office for any of the following reasons:

- Any and all serious and/or life-threatening events which may possibly be reasonably associated, i.e., may reasonably be regarded as caused by, or reasonably be regarded as probably or possibly caused by, the investigational drug used in this protocol, or due to drug administration.
- All fatal and unexpected events regardless of cause or association with study treatment.
- All first occurrence of any "unexpected" (previously unobserved or unreported) toxicity (regardless of Grade).

11.2 Definitions

The following definitions of terms as per Federal Regulations apply to this section:

- ***Serious adverse event***: any experience that is fatal or life threatening, is permanently disabling, requires inpatient hospitalization, or is a congenital anomaly, or overdose.

- ***Associated with the use of the drug***: there is reasonable possibility that the experience may have been caused by the drug or combination of drugs.

- ***Unexpected adverse event***: any adverse event that is not identified in nature, severity, or frequency in the current investigator's brochure or package insert.

11.3 Algorithm for Reporting Adverse Reactions

- Any and all serious and/or fatal or life threatening events which may be associated with the investigational drug used in this protocol, or due to drug administration. (see above):
- Report by telephone or facsimile transmission within 24 hours regarding to the Cancer Center Protocol Office at 802-656-2967.

Person to be contacted:

Paul Horton, Records Coordinator
2nd Floor Medical Alumni Building
Burlington, VT 05405
Phone: 802-656-2967
Fax: 802-656-8788
Email: phorton@zoo.uvm.edu

- Any and all serious and/or fatal or life threatening events which are not associated with the investigational drug used in this protocol or with drug administration should be reported to the Cancer Center Protocol Office **within 5 days.**

- A written report of all adverse effects or experiences and deaths will be submitted by the investigator/co-investigator. In this report, the investigator will advise whether or not the AE is judged to be attributable to the study medication. All such subjects should be followed clinically by the appropriate diagnostic studies. Side effect or subjective symptomatology volunteered by a subject will be noted and recorded as to type and severity on the individual's patient chart. If no side effects are experienced, this also will be reported on the patient chart.

- In turn the Protocol Office will inform the University Institutional Review Board (IRB) and the VCC Protocol Review Committee (PRC) and the FDA if necessary.

12.0 Statistical Considerations

Successful binding of ligand can be viewed as a dichotomous variable and this perspective will be taken relative to the examination of the data. Ligand binding will be examined at two distinct time points and the percent of subjects in whom a ligand is identified at each of the two time points will be initially quantified using exact 95% binomial confidence intervals. A sample size of n = 20 subjects would give rise to a standard error of at most 9.7% for a binomial point estimate under the assumption that the actual binding success rate would be at the 75% or higher level. With a 75% successful binding level, it is anticipated that an expected value of 7.5 out of the first 10 subjects examined would be observed. Since a change in the phage library would be desired if the library were not producing a sufficient rate of binding, we will examine the data in a sequential fashion. The probability of observing four or fewer successful events out of n = 10 subjects equals 2% using a cumulative binomial distribution with individual trial probability of success of 75%. Thus, if four or fewer successful events are observed in the first ten subjects, we will shift to a new phage library. If five or more of the first ten subjects have ligands identified, the next ten subjects will be examined using the same phage library. Since individual subject data are to be obtained at two distinct time points, the experimental design leads to a binary repeated measures setting. Each subject will be considered as a stratum within the context of a conditional logistic regression model. Given the small sample size, an exact procedure will be taken that assumes that the timing effects are conditionally independent of the stratum effects. The software package StatXact will be used to implement examination of the equality of the success rates levels at the two time points. Cross reactivity with other tissue types (total tissue types = 32) will be examined individually for each tissue type using contingency table methods with quantification of the level of cross reactivity using a 95% confidence interval for the odds ratios. Identification of the prevalence of common peptide motifs will be estimated using exact 95% binomial confidence intervals. It is anticipated that a 30% prevalence of a common motif will give rise to a point estimate with a standard error of about 9%.

13.0 Records to be Kept

Data on the tumor characteristics will be collected from the pathology reports, infusion data (amount, batch number) will be recorded, and the molecular consensus sequences of possible binding peptides will be documented.

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15.0 **Informed Consent**

INFORMED CONSENT

Protocol Title: In Vivo Selection of Ligands for Targeted Therapy

Protocol Chairman: David N. Krag, M.D.

Responsible Physician: _____

Sponsor: National Cancer Institute and Vermont Cancer Center, University of Vermont

I, _____, understand that I am being invited to participate in the above research study that has been explained to me by Dr. _____. It has been explained to me that I have melanoma and have been invited to participate in a clinical study to identify new methods of selectively targeting and delivering anticancer drugs directly to a melanoma.

Present treatments for melanoma are often ineffective because they lack the ability to target cancer cells. Specifically, anticancer drugs are injected intravenously and distribute throughout the entire body. The drug itself has no particular preference for concentrating in cancer cells. Therefore the entire body is exposed to the same dose of toxic anticancer drugs. The effect of toxic anticancer drugs on normal non-cancer cells is a limiting factor. This means that the dose of drug given can only be so much. It may take considerably more drugs than the body can tolerate in order to cure a patient of melanoma.

The ultimate goal of this clinical research is to develop a new way to target and selectively concentrate anticancer drugs to cancer cells. We propose to add a small tag, which is a molecule called a peptide, to the anticancer drug. This special tag would allow the anticancer drug to be delivered and concentrated at melanoma cells. In this way, the concentration of drug would be much higher at the cancer cells than normal non-cancer cells.

The immediate goal of this protocol is to develop the tag that will attach to the "address" of cancer cells. The targeting agent (tag) will later be designed to deliver anticancer drugs directly to the address of the cancer cells. Right now we do not know the address of cancer cells. In order to find a tag to cancer cells we propose to intravenously inject several million different tags (or molecules). A small piece of the cancer is removed and the tags that have found their way to the cancer cells (out of the millions injected) and are able to stick at the address of cancer cells will be determined.

Attaching each of the tags to a special agent called a bacterial virus allows my doctor to find the special tag that sticks preferentially to the cancer cells. My doctor is able to find the bacterial viruses and is then able to determine the exact nature (or type) of the tag that sticks to my cancer.

The bacterial viruses (which carry the tag) are living agents that are similar to the human viruses (or germs) that cause the flu and other minor and sometimes serious illnesses in people. These bacterial viruses are special and are not known to infect humans or any other animals. They are not known to cause any human illnesses and may act to protect humans against bacterial infections. They are only able to infect a very small living cell called a bacterium. Many thousands of humans have been previously injected with special bacterial viruses (similar to the ones proposed here) and adverse responses are extremely rare.

Following injection of the special tags, my doctor will remove a small piece of cancer from my body. The size of this piece of cancer will be a cube about than 1/2 an inch on edge. The reason for removing

this piece is to find the special tags that have stuck to my cancer cells. This procedure will be a minor surgical procedure and may involve a small incision and placement of stitches to close the wound. This procedure will be done under local anesthesia. Several hours later another small piece of my cancer will be removed

It is expected that my doctor will find many tags in the cancer specimen that was removed from my body. Some of these tags are there because they truly stick strongly to my cancer cells. These are the tags that my doctor is after. Some of the tags, however, will be just passersby. That is, some of the tags will just happen to be passing though the cancer at the time the small specimen is removed and will not really be sticking to the cancer.

In order to determine which tags truly stick and which do not (or do not stick very well) my doctor will purify the tags found in my cancer and later reinject them into my vein. Just as was done following the first injection, a piece of cancer will be removed, and several hours later another small piece of my cancer will be removed. This time there will be many less types of special tags to bind to my cancer cells and it is more likely that my doctors will be able to identify the tags that stick most strongly to my cancer cells. The entire procedure of injection and biopsies will be repeated a third time. All of the injections and biopsies will be performed within 7 to 14 days.

Before the first injection takes place my doctor may perform a biopsy to have enough of my cancer cells available for later testing. The tags later identified to stick most strongly to my cancer cells will be tested against the first cancer biopsy material to determine how sticky they are.

This entire procedure: 1) biopsy cancer tissue, 2) injection of special tags in my veins and biopsy of cancer tissue, and 3) repeat injection of special tags and repeat biopsy of cancer tissue, is only one set of important steps required before this method of targeting anticancer drugs can be useful in possibly treating cancer. This clinical study is only designed to determine if this method (injection of tags and biopsy of cancer tissue) can find the special tags that stick preferentially to my cancer cells.

In order for this method to be possibly useful in treating my cancer an entire additional set of studies need to be performed. These additional studies involve connecting the special tag to anticancer drugs for special delivery to my cancer cells. It is important for me to understand that these second set of studies will not be performed as part of this clinical study I am being invited to participate in. That means that the findings from this clinical study will not necessarily be useful to me personally in treating my cancer.

BENEFITS AND RISKS:

I understand that I will be monitored very closely by either a nurse, or my doctor, or both, during the time of the injection of the material into my veins. I understand that there is a risk of allergic reaction to the material. Although the risk is very low (less than 1 in a thousand) it is real and may be serious and possibly life threatening. I understand that medications will be immediately available to counteract any allergic reactions, no matter how severe.

I understand that I will have an intravenous catheter placed into one of my veins. This is called an "IV" and it may be uncomfortable since it requires placement of a needle into my vein first. It will remain in my arm for several hours until the entire procedure is complete. I understand that a risk of having an intravenous catheter placed is an infection. This is a small risk but is real. If this should happen I may need to put warm soaks on the area and may need to take an antibiotic.

I understand that I will have up to seven biopsies of my cancer tissue. Each one of these biopsies will be small but will involve a small surgical procedure. I understand that local anesthesia will be injected

around the biopsy site to numb the tissue. I may need to have stitches placed. There is a small risk of infection as there is with any surgery. If this should happen I may need to put warm soaks on the area and may need to take an antibiotic.

I understand that this clinical study will be of no immediate or direct benefit to me. It may in the future lead to important findings that may benefit others. There is a remote possibility that if this study is successful I may participate in subsequent clinical studies designed to test the anticancer effectiveness of this technique.

ALTERNATIVE TREATMENTS:

I understand that my therapy will not be lessened by participation in this trial. I understand that this clinical study is not a therapeutic study and therefore not related to my treatments.

I have discussed the above facts with my physician and have been given the opportunity to ask questions which have been answered to my satisfaction. I understand that my physician will answer any questions that I might have.

COSTS AND PAYMENTS:

The injection of the special tagged material and the surgical biopsies will not be billed to me. Other medications and all physicians' and hospital costs related to my regular treatments will be charged to me in the same fashion as if I was not part of this study. I will receive no monetary compensation for my participation in this study.

CONFIDENTIALITY:

I understand that a record of my progress will be kept in a confidential form at the Vermont Cancer Center, University of Vermont (VCC/UVM). I understand that the results of this study may eventually be published and that information may be exchanged between medical investigators, but that patient confidentiality will be maintained. There is a possibility that my medical record, including identifying information, may be inspected and/or photocopied by qualified representatives from VCC/UVM, the National Cancer Institute or other Federal or state government agencies in the ordinary course of carrying out their governmental functions. If my record is used or disseminated for government purposes, it will be done under conditions that will protect my privacy to the fullest extent possible consistent with laws relating to public disclosure of information and the law-enforcement responsibilities of the agency.

RIGHT TO WITHDRAW:

I understand that I am free to refuse to participate in this trial or to withdraw at any time and that my decision will not adversely affect my care at this institution or cause a loss of benefits to which I might otherwise be entitled.

COMPENSATION FOR ILLNESS OR INJURY:

I understand that it is not the policy of the University of Vermont or Fletcher Allen Health Care to provide payment or free medical treatment for injury resulting from research. I understand that I may contact Dr. David Krag, One South Prospect St. (802-656-2262) for more information about this study or Nancy Stalnaker, the Institutional Review Board Administrator, 231 Rowell Building (802-656-4067) for information regarding my rights as a research subject or for information about how to proceed should I believe that I have been injured as a result of my participation in this study.

VOLUNTARY CONSENT:

I have been given an opportunity to discuss this trial with the physician(s) conducting it and I understand that I may ask further questions and that I may withdraw from the study at any time. Withdrawal from the study will not prejudice my further care at the Vermont Cancer Center or Fletcher Allen Health Care. I agree to participate in this study and I acknowledge that I have received a signed copy of this consent form.

_____ _____

Signature of Patient Date

_____ _____

Signature of Witness Date

_____ _____

Signature of Physician Date

Protocol Chairman:

David Krag, M.D.
Surgical Associates
One South Prospect
Burlington, VT, 05401
802-656-2262

APPENDIX I

NCI COMMON TOXICITY CRITERIA

TOXICITY (Adverse Event)	GRADE 0	GRADE 1 (Mild)	GRADE 2 (Moderate)	GRADE 3 (Severe)	GRADE 4 (or Life-Threatening)
BLOOD/BONE MARROW					
WBC	≥ 4.0	3.0 - 3.9	2.0 - 2.9	1.0 - 1.9	< 1.0
PLT	WNL	75.0 - normal	50.0 - 74.9	25.0 - 49.9	< 25.0
Hgb	WNL	10.0 - normal	8.0 - 10.0	6.5 - 7.9	< 6.5
Granulocytes/ Bands	≥ 2.0	1.5 - 1.9	1.0 - 1.4	0.5 - 0.9	< 0.5
Lymphocytes	≥ 2.0	1.5 - 1.9	1.0 - 1.4	0.5 - 0.9	< 0.5
LIVER					
Bilirubin	WNL	—	< 1.5 x N	1.5 - 3.0 x N	> 3.0 x N
Transaminase (SGOT, SGPT)	WNL	≤ 2.5 x N	2.6 - 5.0 x N	5.1 - 20.0 x N	> 20.0 x N
Alk Phos or 5' nucleotidase	WNL	≤ 2.5 x N	2.6 - 5.0 x N	5.1 - 20.0 x N	> 20.0 x N
GASTROINTESTINAL					
Nausea	none	able to eat reasonable intake	intake significantly decreased, but can eat	no significant intake	—
Vomiting	none	1 episode in 24 hours	2-5 episodes in 24 hours	6-10 episodes in 24 hours	> 10 episodes in 24 hours or requiring parenteral support
Diarrhea	none	increase of 2-3 stools/day over pre-Rx	increase of 4-6 stools/day, or nocturnal stools, or moderate cramping	increase of 7-9 stools/day, or incontinence, or severe cramping	increase of ≥10 stools/day, or grossly bloody diarrhea, or need for parenteral support
Stomatitis	none	painless ulcers, erythema, or mild soreness	painful erythema, edema, or ulcers, but can eat	painful erythema, edema, or ulcers, and cannot eat	requires parenteral or enteral support
KIDNEY/BLADDER					
Creatinine	WNL	< 1.5 x N	1.5 - 3.0 x N	3.1 - 6.0 x N	> 6.0 x N
Proteinuria	no change	1+ or <0.3 g% or < 3 g/l	2 - 3+ or 0.3 - 1.0 g% or 3 -10 g/l	4+ or > 1.0 g% or > 10 g/l	nephrotic syndrome
Hematuria	neg	micro only	gross, no clots	gross + clots	requires transfusion

APPENDIX I (Continued)

TOXICITY (Adverse Event)	GRADE 0	GRADE 1 (Mild)	GRADE 2 (Moderate)	GRADE 3 (Severe)	GRADE 4 (or Life-Threatening)
HEART/LUNGS					
Cardiac dysrhythmias	none	asymptomatic, transient, requiring no therapy	recurrent or persistent, no therapy required	requires treatment	requires monitoring; or hypotension, or ventricular tachycardia, or fibrillation
Cardiac function	none	asymptomatic, decline of resting ejection fraction by less than 20% of baseline value	asymptomatic, decline of resting ejection fraction by more than 20% of baseline value	mild CHF, responsive to therapy	severe or refractory CHF
Cardiac–ischemia	none	non-specific T-wave flattening	asymptomatic, ST and T wave changes suggesting ischemia	angina without evidence for infarction	acute myocardial infarction
Cardiac–pericardial	none	asymptomatic effusion, no intervention required	pericarditis (rub, chest pain, ECG changes)	symptomatic effusion; drainage required	tamponade; drainage urgently required
Pulmonary	none or no change	asymptomatic, with abnormality in PFT's	dyspnea on significant exertion	dyspnea at normal level of activity	dyspnea at rest
Weight gain/loss	< 5.0%	5.0 - 9.9%	10.0 - 19.9%	≥ 20.0%	—
BLOOD PRESSURE					
Hypertension	none or no change	asymptomatic, transient increase by greater than 20 mm Hg (D) or to > 150/100 if previously WNL; no treatment required	recurrent or persistent increase by greater than 20 mm Hg (D) or to > 150/100 if previously WNL; no treatment required	requires therapy	hypertensive crisis
Hypotension	none or no change	changes requiring no therapy (including transient orthostatic hypotension)	requires fluid replacement or other therapy	requires therapy and resolves within 48 hours of stopping the agent	requires therapy for > 48 hours after stopping the agent

APPENDIX I (Continued)

TOXICITY (Adverse Event)	GRADE 0	GRADE 1 (Mild)	GRADE 2 (Moderate)	GRADE 3 (Severe)	GRADE 4 (or Life Threatening)
NEUROLOGIC					
Neuro–sensory	none or no change	mild paresthesias, loss of deep tendon reflexes	mild or moderate objective sensory loss; moderate paresthesias	severe objective sensory loss or paresthesias that interfere with function	—
Neuro–motor	none or no change	subjective weakness; no objective findings	mild objective weakness without significant impairment of function	objective weakness with impairment of function	paralysis
Neuro–cortical	none	mild somnolence or agitation	moderate somnolence or agitation	severe somnolence, agitation, confusion, disorientation, or hallucinations	coma, seizures, toxic psychosis
Neuro–cerebellar	none	slight incoordination dysdiakokinesis	intention tremor, dysmetria, slurred speech, nystagmus	locomotor ataxia	cerebellar necrosis
Neuro–mood	no change	mild anxiety or depression	moderate anxiety or depression	severe anxiety or depression	suicidal ideation
Neuro–headache	none	mild	moderate or severe but transient	unrelenting and severe	—
Neuro–constipation	none or no change	mild	moderate	severe	ileus .> 96 hours
Neuro–hearing	none or no change	asymptomatic, hearing loss on audiometry only	tinnitus	hearing loss interfering with function but correctable with hearing aid	deafness not correctable
Neuro–vision	none or no change	—	—	symptomatic subtotal loss of vision	blindness

APPENDIX I (Continued)

TOXICITY (Adverse Event)	GRADE 0	GRADE 1 (Mild)	GRADE 2 (Moderate)	GRADE 3 (Severe)	GRADE 4 (or Life Threatening)
DERMATOLOGIC					
Skin	none or no change	scatter macular or papular eruption or erythema that is asymptomatic	scatter macular or papular eruption or erythema with pruritus or other associated symptoms	generalized symptomatic macular, papular, or vesicular eruption	exfoliative dermatitis or ulcerating dermatitis
Palmar-Plantar Erythro-dysesthesia	no symptoms	Mild erythema, swelling, or desquamation not interfering with daily activities.	Erythema, desquamation, or swelling interfering with, but not precluding, normal physical activities; small blisters or ulcerations less than 2 cm in diam.	Blistering, ulceration, or swelling interfering with walking or normal daily activities; cannot wear regular clothing.	Diffuse or local process causing infectious complications, or a bed ridden state or hospitalization.
Allergy	none	transient rash, drug fever < 38°C, 100.4°F	urticaria, drug fever = 38°C, 100.4°F, mild bronchospasm	serum sickness, bronchospasm requiring parenteral medications	anaphylaxis
Alopecia	no loss	mild hair loss	pronounced or total hair loss	—	—
METABOLIC					
Hyperglycemia	< 116	116 - 160	161 - 250	251 - 500	> 500 or ketoacidosis
Hypoglycemia	> 64	55 - 64	40 - 54	30 -39	< 30
Amylase	WNL	< 1.5 x N	1.5 - 2.0 x N	2.1 - 5.0 x N	> 5.1 x N
Hypercalcemia	< 10.6	10.6 - 11.5	11.6 - 12.5	12.6 - 13.5	> 13.5
Hypocalcemia	> 8.4	8.4 - 7.8	7.7 - 7.0	6.9 - 6.1	≤ 6.0
Hypomagnesemia	> 1.4	1.4 - 1.2	1.1 - 0.9	0.8 - 0.6	≤ 0.5

APPENDIX I (Continued)

TOXICITY (Adverse Event)	GRADE 0	GRADE 1 (Mild)	GRADE 2 (Moderate)	GRADE 3 (Severe)	GRADE 4 (or Life Threatening)
COAGULATION					
Fibrinogen	WNL	0.99-0.75 x N	0.74-0.50 x N	0.49-0.25 x N	≤ 0.24 x N
Prothrombin time	WNL	1.01-1.25 x N	1.26-1.50 x N	1.51-2.00 x N	> 2.00 x N
Partial thrombo-plastin time	WNL	1.01-1.66 x N	1.67-2.33 x N	2.34-3.00 x N	> 3.00 x N
Hemorrhage (Clinical)	none	mild, no transfusion	gross, 1-2 units transfusion per episode	gross, 3-4 units transfusion per episode	massive, >4 units transfusion per episode

Source (modified from): National Institute of Health, National Cancer Institute, Cancer Therapy Evaluation Program, Bethesda, Maryland 20892

Chills (rigors)	none	any rigor, mild	rigors requiring medication	rigors not controlled by medication	—

Appendix II

DATA COLLECTION FORMS

Registration & Eligibility Checklist
VCC Confirmation of Registration
Adverse Event Report

In Vivo Selection of Ligands for Targeted Therapy
Patient Registration Form (Page 1)

PLEASE PRINT

Surgeon Of Record: _____

Contact Person: _____

Phone Number: _____ FAX :_____

Patient Name: _____

Birth Date: ____ / ____ / ____ Date of Diagnosis: ____ / ____ / ____

Signed Consent Attached? ____ Yes ____ No

FAX BOTH REGISTRATION PAGES AND THE ENTIRE CONSENT FORM TO:
802-656-1987

PATIENT WILL NOT UNDERGO PROCEDURE UNLESS THIS SHEET IS FAXED BACK TO YOU WITHASSIGNED ID NUMBER.

BELOW LINE FOR OPERATIONS CENTER USE ONLY

Assigned Patient ID Number: _____

Date of Registration: ____ / ____ / ____

CHRMS #: _____

Registrar: _____

Checklist

Registration form legible and complete ___
Consent form legible and complete ___
Eligibility criteria are all checked "Y" ___
Consent form is correct for study ___
Consent form has not expired ___

VCC registration form submitted ____

In Vivo Selection of Ligands for Targeted Therapy

Patient Registration Form (Page 2)

Eligibility Criteria

Patient Name: _____ Date of Birth: _____

1. The patient has metastatic or locally advance primary or recurrent melanoma that is not considered curable by conventional therapy. Y ___ N ___
2. The patient has superficial cancer nodules or mass amenable to biopsy with minor surgery. Y ___ N ___
3. The patient has a Karnofsky status ≥ 70 and a life expectancy ≥ 4 months. Y ___ N ___
4. The patient has undergone an informed consent process. Y ___ N ___
5. The patient is not pregnant. Y ___ N ___
6. The patient does not have any other serious illness, other than that treated by this study Y ___ N ___
7. The patient is 30 to 70 years of age. Y ___ N ___
8. The patient has no evidence of extensive pulmonary metastases. Y ___ N ___
9. The patient has no clinical symptoms suggestive of brain metastases. Y ___ N ___

All statements must be checked "Y" or "N/A" for entry into the trial.

Note: this sheet does not constitute source documentation. The above information must be included elsewhere in the patient's hospital chart in a recognized source document.

Signature of Physician: _____ Date: _____

VCC CONFIRMATION OF REGISTRATION

In Vivo Selection of Ligands for Targeted Therapy

Physician:_____

Patient Name:_____
(Please print) Last First Middle

Hospital Chart #:_____Social Security # _____

Race: _____ Sex: Male Female Date of Birth ____/____/____
(1-White, 2-Hispanic, 3-Black, 4-Oriental,
5-Native Hawaiian, 6-Native American, 7-Indian,
8-Filipino, 9-Other, 10-Patient refusal, 1-Institution
refusal, -1-unkown)

Method of Payment_____ Zip Code:_____
(1-Private Insurance,2-Medicare, 3-Medicare
and private insurance, 4-Medicaid, 5-Medicaid and
Medicare, 6-Military or Veterans Administration
Sponsored, 7-Self Pay (no insurance), 8-No means of
payment (no insurance), -1-unknown)

Eligibility Criteria:- See protocol checklist
_____Patient Eligible? (1-no; 2-yes, all requirements confirmed)

If assignment is necessary from VCC, please fax to Donna Silver 802-656-8788 to get registration.

If different levels list here as 1,2 3.

Patient Study Number _____
Level Assigned _____
Date Registered_____
Registrar _____

VCC Registration Form

REPORT OF ADVERSE EVENTS AND/OR UNANTICIPATED PROBLEMS

All items on this form must be completed by the principal/co-investigator. Please attach any additional information.

Submit completed form to:

Cancer Center Protocol Office
2nd Floor Medical Alumni Building
Burlington, VT 05405

FOR VCC USE ONLY
Date of Notification
Initial Contact ___/___/___
FU Contact ___/___/___

CHRMS #: _____ PRINCIPAL INVESTIGATOR: David N. Krag, MD

PROTOCOL NUMBER AND TITLE: In Vivo Selection of Ligands for Targeted Therapy

Patient Identification Number: _____Date of event/problem: _____

Brief description of event/problem (please do not indicate "see attached" as a response):

Did event/problem occur here? Yes _____ No _____

Was event/problem related to protocol? Yes _____ No _____ Unsure _____
If yes, how?

Have there been similar events/problems reported here? Yes _____ No _____
Elsewhere? Yes _____ No _____
If yes to either, explain:

Was the protocol discontinued for this subject? Yes _____ No _____ Unknown _____

Was further treatment required? Yes _____ No _____
If yes, explain:

Does the protocol need to be modified as a result of this report? Yes _____ No _____
If yes, explain:

Does the consent form need to be modified as a result of this report? Yes _____ No _____
If yes, explain:

Note: You should keep a copy of this completed form as this information *must* be included in your summary of events/problems encountered during the indicated time period of *your next continuing* review.

Chemistry, Manufacturing and Control Information

Safety:

We do not expect the screening procedure to cause toxicity as bacteriophage have been injected intravenously in humans and even neonates for over 30 years in approximately 3000 patients with essentially no side effects. This is extensively reported in the literature [2, 4, 5, 7, 18]. Phage are injected into humans IV routinely for analysis of antibody responses.

There has also been extensive use of over 250 strains of bacteriophage, including 39 which infect *Escherichia* bacteria, which were administered orally or locally for treatment of infection[10-16]. Not only were "side effects" described as "extremely rare" (3 allergic responses out of 138, with no prior endotoxin testing), the phage treatments were often effective in eliminating the bacterial infection.

Bacteriophage are known to specifically infect only bacteria, and each bacteriophage strain infects only a very narrow range of bacterial species. Therefore, the infection of eukaryotic cells by bacteriophage is unlikely. One group has used filamentous phage to transfect DNA into mammalian cells. Single-stranded DNA was ultimately expressed by the mammalian cells, demonstrating that single-stranded DNA can be converted into double-stranded DNA inside mammalian cells. However, the DNA that was expressed was DNA coding for mammalian proteins, not phage proteins. (The DNA was packaged inside phage particles.) Importantly for safety considerations, the phage particles did not transfect cells at all in the absence of transfection agents such as DEAE-dextran or lipopolyamine, which would not be present in an in vivo situation. Furthermore, these experiments looked at expression only at about one week after transfection. Whether the expression was transient, or more permanent, suggesting incorporation of the DNA into the host cell genome, was not addressed[20, 21]. The presence of phage DNA in monkey cells was examined following ϕX174 phage-injection (see Ochs IND, Appendix). Several months after injection using a labeled DNA probe, no phage DNA was detected (albeit PCR was not used). Presumably, incorporation of phage DNA into eukaryotic DNA can be detected by PCR. While we have not performed PCR analysis in the present studies, we have tissues stored for that purpose in the event that PCR analysis for phage DNA is judged to be necessary.

Dr. Hans Ochs, an immunologist who is one of the pioneers of using phage ϕX174 injection for human antibody response analysis, is a collaborator on this project. Dr. Ochs' group has had an IND from the FDA for over 20 years for this procedure and has extensive experience with IV injections of bacteriophage in humans. He also has extensive experience with the immunological consequences of intravenous administration of bacteriophage. IND information regarding these studies and his letter of support are included in the Appendix. We will have his expertise available to us for consultations on human phage injections, and he will provide us with his strain of phage for library construction, if necessary. Not only has the work of Ochs and his colleagues been documented for non-toxicity (see Appendix), their experiments give us an accurate estimate of the time we have available to screen before we expect elimination of injected phage by the immune system. Our library is constructed in a strain of bacteriophage different than the strain used for antibody response analysis, although they both infect *E. coli* exclusively. Ochs uses a lytic icosahedral bacteriophage strain, while our libraries are made with filamentous phage which do not lyse the cells they infect. Filamentous phage injection is not likely to cause toxicity, as there have been numerous reports of the injection of filamentous phage into mice for purposes such as

hybridoma development. There are also several groups investigating the use of filamentous phage particles as potential vaccine delivery agents, with numerous preliminary studies in mice. At least one of these studies reports subcutaneous injection of a large number (6.7×10^{12}) of phage particles. Although toxicity was not explicitly addressed[19], no toxicity was reported, and the mice survived at least 3 to 4 days when they were sacrificed in order to harvest spleen cells for hybridoma development. Additionally, all but one of the mice (n=31) injected with peptide-phage in our mice toxicity studies survived to the experimental endpoint, either 3 days or 3 weeks. The one mouse that did not survive probably died as a result of surgical/anesthesia complications. Our phage will also bear peptides, which may present complications particularly after amplification from tumor tissue, which will have normal tissue components. However, the absolute mass of peptide we propose to inject will be extremely small, approximately 17 picograms to 17 nanograms. Nevertheless, the proposal has been designed to detect any possible toxicities caused by these peptides. Most of the toxicity studies performed by our laboratory, as described in this IND application, indicate that in vivo screening is quite safe, at least in mice. The only significant pathology we detected washepatitis, found in 3 out of 31 livers (from phage-injected mice) that were examined by the project pathologist. (See Section 8: Pharmacology and Toxicology). The livers with hepatitis were all from the same cohort of FVB mice. No hepatitis was found in any other strain. We suspect that these mice had hepatitis prior to injection with peptide-phage. However, because there is no way to confirm this, we will monitor the liver function of patients during Phase I trials.

During the manufacturing of our peptide-phage, Triton X-114 is employed (using the method of Aida and Pabst[1] to remove endotoxins. Aida et al showed that the amount of Triton X-114 remaining in the preparation is 0.018% which they found detrimental to the cellular activity of neutrophils. Our preparation will be in a final volume of 20 ml. Prior to administration this will be diluted in 250 ml of saline and infused over 10 minutes. (See clinical protocol.) This 13.5-fold dilution will decrease Triton X-114 to 0.00133%. Assuming an average blood volume of 5 liters, the product will undergo another 20-fold dilution once it is in the bloodstream, or 0.00007%. Aida's group showed only a slight decrease in neutrophil cellular activity with 0.002% Triton X-114. Our preparation, once in the blood stream, will be 28-fold more dilute. Therefore, we feel this poses little risk to humans.

The peptide-phage that we will be using for Phase I clinical trials will be nearly identical to that used in our animal studies. We will not need to use polyethylene glycol (PEG) a second time (see details under Drug Product—Manufacturing). PEG is used to concentrate the material, but since we will not have the volume restrictions we had with mice, the second PEG concentration will not be necessary. Since our endotoxin testing has been done on phage preparations after two PEG concentrations, we will (prior to injections into patients) test our product again without the second PEG step to make certain that the second PEG step is not somehow necessary for decreasing endotoxin content.

Drug Substance: Phage Displayed Random Peptide Libraries (RPL)

Physical, chemical or biological characteristics

The phage-displayed RPL which will be used in this study are made using filamentous phage. Filamentous phage have been used routinely for many years now in the laboratory for cloning and sequencing. The genomes of several strains of these phage (ϕ) were completely sequenced almost 20 years ago and were in fact, among the first complete genomes sequenced. They have been completely characterized in that the function of all 11 genes are well understood.

The best characterized filamentous phage strains are M13, f1, and fd. The genomes of these three phage strains are highly homologous (greater than 98% identical at the nucleotide level) and may be regarded as identical for most purposes. We have constructed libraries in phage vector systems derived from both fd and f1 strains of phage. The strain of filamentous phage used in the studies described in this IND application is fd-tet, which allows infected bacteria to grow in the presence of tetracycline. All filamentous phage infect only male *E. coli* strains as they enter the *E. coli* cells via an F-pilus coded for by an F-plasmid. Because of this dependence on an F-pilus, M13, f1, and fd are sometimes referred to as Ff phage.

Filamentous phage are long thin particles, approximately 6.5 nm in diameter and about 930 nm in length. Genetically engineered particles can be longer. They are composed of a tubular, helical array of approximately 2700 copies of the major coat protein (the product of gene VIII) surrounding the single-stranded, covalently closed, circular DNA genome. The wild-type genome is approximately 6400 bases. There are four minor coat proteins, several copies of two of the minor coat proteins (pIII and pVI) are located at one end of the particle, while several copies of the other two minor coat proteins (pVII and pIX) are located at the opposite end of the particle. In particular, the pIII minor coat protein is often used in library construction to display peptides or proteins from its N-terminus, which is exposed to the environment outside the particle. There are 3-5 copies of pIII located at one end of the phage particle. The normal function of pIII, also called the "pilot" protein, is to bind to the end of the bacterial F-pilus as the first step of infection into the host cell. The major coat protein, pVIII, is also commonly used in the construction of phage-displayed libraries. The libraries used in these studies were constructed in a pIII system.

A quantity of 0.67×10^{11} Ff particles weighs about 1 ug. This is about two-thirds the density of ϕX174 phage particles (1×10^{11} virions in 1 ug). Approximately 1 mg of DNA is present in 2×10^{14} virions. There is about six times more viral protein than viral DNA by mass.

Filamentous phage particles bind to the pili of male strains of *E. coli* and are retracted into the cell. Once inside the cell, the protein coat is released from the single stranded covalently closed circular phage DNA molecule. The single-stranded molecule is converted by host enzymes into a double-stranded form called the replicative form (RF). The RF multiplies like a plasmid to a low copy number inside the *E. coli* cell. About ten minutes after infection, copies of single-stranded DNA are produced from the RF DNA, and coat proteins (coded by phage DNA) are produced and assemble at the *E. coli* cell membrane. The single-stranded DNA molecules are secreted through the membrane and are coated by the coat proteins as they pass through the membrane, to form intact phage particles, which are secreted into the media. Infected cells grow more slowly than wild-type bacteria, but are not killed by the infection and secretion process. Phage that display peptides on the gene III coat proteins are, in general, just as capable of infection and amplification in *E. coli* as wild-type phage. Even large proteins such as single chain antibodies can be displayed by phage without appreciably affecting their ability to infect and multiply.

The libraries we have constructed display peptides from 11-22 amino acids in length. Most libraries have cysteines placed in the displayed peptides to enable formation of a loop of 8-12 amino acids. Our libraries contain 2×10^7 to 10×10^{10} different peptides.

Name and address of manufacturer

David Krag, MD
University of Vermont
Given E-309
Burlington, Vermont 05405

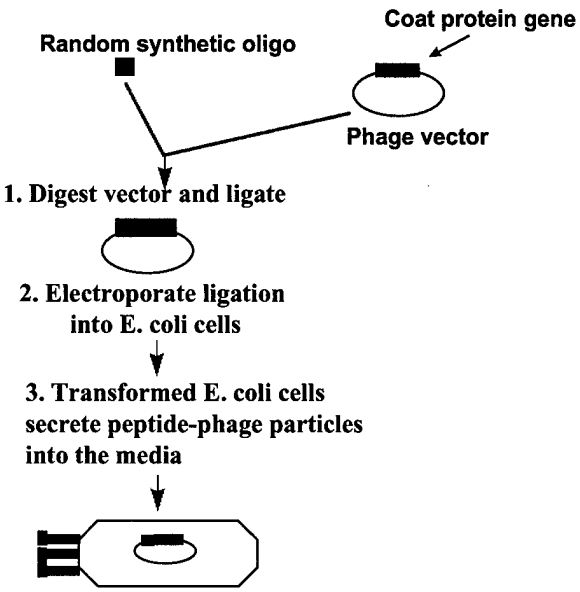
Method of preparation

The library was constructed to contain a variable 9-amino acid peptide flanked by cysteine residues inserted into the Gene III protein of the phage fUSE5[6, 17]. The phage were quantified by titering (refer to Appendix for Phage Titering Protocol).

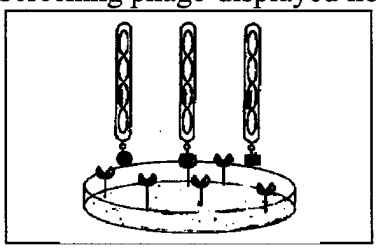
Random peptide phage-display technology

This technology consists of the construction of huge libraries of peptides displayed on phage particles (libraries constructed in our laboratory contain $2x10^7$ to $1x10^{10}$ different peptide-phage clones). These libraries are a rich source of small peptide ligands. Peptide ligands to a wide variety of targets can be identified by a simple affinity selection (screening) procedure.

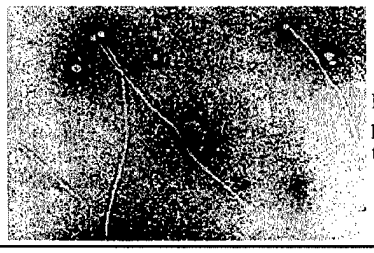
Construction of phage-displayed peptide libraries



Screening phage-displayed libraries



1. Bind target to a matrix.
2. Incubate peptide-phage library with target.
3. Wash away non-binding peptide-phage.
4. Elute peptide-phage which bind.
5. Amplify binding peptide-phage in E. coli cells.
6. Sequence DNA of binding clones to deduce the amino acid sequence of the binding peptides.
7. Analyse amino acid sequences of clones for consensus sequences.
8. Design biased libraries based on the consensus to obtain tighter binders.



Electron micrograph of phage (filaments) binding to target (beads)

Acceptable limits and analytical methods to assure identitiy, strength and quality
Phage are identified by detection of colonies growing on Luria-Bertani media (LB) agar, pH 7.0, supplemented with tetracycline at a concentration of 40 μg/ml. Because the vector used to construct the libraries has a tet-resistant gene and filamentous phage do not lyse the host bacterium, one colony represents one infectious phage particle or transducing unit (TU). In the phage-display system we used for the present studies (fUSE 5, George Smith), only about 1 in 20 phage actually infect *E. coli* [9].

The quality of the phage is assured by sequencing the DNA. DNA sequencing determines the percent of phage with the correct insert.

Stability of substance
Phage particles are stable and infective for years when stored at 4°C. However the stability of the peptides displayed on the phage particles unknown and is likely to vary from peptide to peptide. (We have preliminary data on one peptide showing stability to 3 weeks.) The substance will be stored at 4°C prior to use in patients and will be used as soon as possible following the preparation of the peptide library.

Drug Product:

Components used in manufacture
The following components are used in the production of our peptide-phage. (Please refer to the Appendix for a more detailed description.) While all of these components are used in the production, the primary components, (used for diluting the product throughout the production) are PBS and DPBS, pH 7.3.

2xYT Media
DPBS (Dulbecco's Phosphate Buffered Saline), pH 7.3, Sigma Product Number: D 8537
EPI (Eukaryotic Protease Inhibitors), Sigma Product Number: P 8340
PBS (Phosphate Buffered Saline), pH 7.3
PEG (Polyethylene Glycol in Sodium Chloride)
PPI (Prokaryotic Protease Inhibitors), Sigma Product Number: P 8465
Triton X-114 (Octylphenoxypolyethoxyethanol)

Name and address of manufacturer
David Krag, MD
University of Vermont
Given E-309
Burlington, Vermont 05405

Product preparation
Filamentous peptide-phage are prepared from *E. coli* cultures grown overnight on 2xYT media agar plates (see Reagents Used in Peptide-Phage Production in the Appendix). The phage particles are resuspended in phosphate buffered saline with prokaryotic protease inhibitors (PBS-PPI) by "sweeping" the agar with an angled glass rod. The phage suspension is centrifuged twice to remove bacterial cells and filtered with a 0.22 um polyethersulfone membrane to completely remove any remaining *E. coli* cells. The phage are concentrated by precipitation with polyethylene glycol (PEG). The resulting pellet is resuspended in fresh PBS-PPI and the phage suspension is passed through a 0.45 μm cellulose acetate filter. (Refer to Phage Amplification and Harvesting Protocol in the Appenix for further details.) Endotoxins are removed from the

preparation by performing three 1% (v/v) Triton X-114 extractions. The phage are concentrated with PEG again and the resulting pellet is resuspended in PBS-PPI. The phage suspension is shaken 10 min at 200 rpm on ice, followed by centrifugation. The supernatant containing the peptide-phage is passed through a 0.45 μm cellulose acetate filter, followed by passage through a pyrogen-free 0.2 μm cellulose acetate filter to sterilize the preparation. (Refer to Appendix for Endotoxin Purification Procedure.)

According to FDA guidelines, establishment of the sterility of any preparation to be injected into humans must be performed by inoculation of the product into Fluid Thioglycollate Media and Tryptic Soy Broth. We have performed these sterility tests exactly as described in the Code of Federal Regulations (21CFR610.12) on representative preparations. These tests have confirmed the sterility of our preparations, as expected after filtration through a pyrogen-free 0.2 μm cellulose acetate filter.

Although the sterilization filtration technique takes only a minute, the FDA guidelines for asserting sterility takes 14 days. This 14 day waiting period is not compatible with the screening experiments we have proposed for 2 reasons:

A) In our experience and judgement, when performing phage-display RPL screening, it is optimal to prepare a fresh batch of a phage-displayed library to optimize complexity of libraries, as some of the displayed peptides within the library may be more susceptible to degradation than other peptides. Ideally we would like to be able to establish proof of sterility, lack of endotoxins, and any other parameters necessary, within a few hours. It is possible that some of these difficulties may be avoided by freezing peptide-phage preparations. However, the freezing process may also compromise the stability of the peptides and therefore, the complexity of the libraries. It is possible that all the peptides which may bind to tumor targets may be stable for 14 days. This may be elucidated as the study progresses. Furthermore, freezing or other storage methods which would avoid peptide degradation for 14 days, will not address the problem raised in point B below.

It may be possible to establish a large batch of libraries used for *initial* screenings only if a storage method which allows the displayed peptides to retain their tumor binding activity can be developed.

B) To perform 3 screens in one person, the screens will probably need to be performed in about 7 days, in order to avoid rejection of phage by the immune system, as a detectable IgM response to injected phage typically begins to develop in humans in about 7 days. (Ochs' IND, Appendix). Serial screening in one person may be the most optimal way to identify tumor-homing peptides, and may well be the only way to identify tumor-specific targets which are individual to a given patient. Therefore, to perform three screens in one patient, a 14 day waiting period for results of sterility testing will probably not be possible.

Endotoxin testing

We have become expert in conducting the Limulus Amebocyte Lysate (LAL) gel clot assay (Charles River Endosafe) for the presence of endotoxins, and in utilizing the meticulous techniques necessary to keep reagents endotoxin-free. All reagents and liquid handling materials must be endotoxin-free. While it is usually easier to purchase items, especially plasticware, certified endotoxin-free, it is possible to destroy endotoxins on glassware and metal by autoclaving, followed by dry heat at 210°C for 3 hours. As per discussions with Stephanie Simek of the FDA, the maximum amount of endotoxin allowed in a substance that will be injected IV into humans is 10 endotoxin units (EU) per kg of patient weight per day. Using a LAL reagent

with a sensitivity of 0.25 EU/ml, we determined that our initial preparations contain roughly 10^5 times more endotoxin than is permissible. Using the Triton X-114 extractions mentioned above, we have decreased the amount of endotoxins in our phage preparations to FDA permissible levels. (see below for data)

Results from LAL endotoxin testing can be obtained in about one hour, and so does not have the same difficulties regarding testing time as with sterility testing.

Additionally, we have performed a LAL gel clot assay according to the manufacturer's instructions to check for interfering substances in our preparation that might inhibit the gel clot reaction. We found no evidence of interference, as shown below.

Test for Product Inhibition Using Charles River Endosafe Assay

From Endosafe insert: "The easiest method to determine the non-inhibitory product concentration is to prepare a series of increasing dilutions of the product containing a 2λ endotoxin concentration. Assay this series as well as a series of the product diluted with water."

04/21/00 LAL Assay for Product Inhibition (λ =0.25 EU/ml)

Tube#	Endotoxin Standards in LAL Reagent Water	Results
1	0.0625 EU/ml	-
2	0.125	-
3	0.25 (1λ)	-
4	0.5 (2λ)	+
	Phage (from 4/3/00 input) and 2λ Endotoxin	
5	1:4 in LAL reagent water	+
6	1:10	+
7	1:20	+
8	1:40	+
9	1:100	+
	Phage alone (from 4/3/00 input)	
10	1:4 in LAL reagent water	+
11	1:10	+
12	1:20	+
13	1:40	-
14	1:100	-
	Tap Water (positive control)	
15	neat	+

Results show no inhibition of the endotoxin test by the phage preparation as demonstrated by the positive results in sample tubes 8-9 (1:40 and 1:100 dilutions of sample) that were spiked with2λ of endotoxin vs. the negative results in tubes 13-14 (1:40 and 1:100 dilutions of sample respectively) that were not spiked.

Additionally, the "phage alone" is shown to be <0.5 EU/ml at a 1:40 dilution.

Final volume of phage prep on 4/3/00 was 2.5 ml. If FDA endotoxin limit=10 EU/kg/day, then the limit for an average 70 kg individual = 700 EU/day.

$$\frac{700\ \text{EU}}{2.5\ \text{ml}} = 280\ \text{EU/ml limit}$$

Product was positive for endotoxin between 1:20 and 1:40 dilution, or 10-20 EU/ml (based on 0.5 EU/ml positive standard), well within FDA limits, even for lighter weight patients.

Particle characterization

In our pre-IND conference call with the FDA, we discussed whether we need to characterize our peptide-phage for particle size and homogeneity, and is so, how. This has not yet been resolved.

Placebo

No placebos are planned for phase I trials.

Labeling for Drug Product*

Study ID #: _____

Name of Patient: _____
MRN: _____
Substance: Peptide-Phage
Lot #: _____
Transducing Units: _____
Dose #: _____

Caution:
New Drug – Limited by Federal (or United States) law to investigational use.

*** Not actual size**

Exclusion from Environmental Assessment

We claim categorical exclusion (under 21 CFR 25.31[e}]) for the studies under this IND. To our knowledge, no extraordinary circumstances exist.

Pharmacology and Toxicology Information

- **Individuals evaluating safety of drug product**

  Dr. David Krag, oncological surgeon

  David Krag, MD has extensive experience with both laboratory and clinical oncology research. He is the PI for a large clinical trial currently underway (anticipated accrual 5200 patients). He also has extensive experience with administration of biological agents in the form of radiolabeled antibodies. He has also developed technology for administration of lymph node targeting agents which involved injection of a variety of radiopharmaceuticals. He is skilled at the detection and management of any potential side effect.

  Dr. Donald Weaver, project pathologist

  Donald L. Weaver, MD, is an Associate Professor of Pathology in the College of Medicine at the University of Vermont and a Board Certified Pathologist practicing surgical pathology at Fletcher Allen Health Care, the University's affiliated teaching hospital. Dr. Weaver's primary practice interest is in Breast Pathology; however, in his surgical pathology practice he is exposed to a broad variety of pathology from all sites of the human body. Although not a veterinary pathologist, Dr. Weaver has occasionally reviewed histologic sections of research animals for other University faculty and reviewed diagnostic veterinary biopsies from tumors and masses in domestic pets, usually cats or dogs, for diagnosis of malignancy. Dr. Weaver's research interest is in Breast Cancer prognosis and predictors of therapeutic response.

- **Where studies were done**

  The following portions of the study were performed in the Vermont Cancer Center at the University of Vermont College of Medicine: phage amplification; mice injections, surgery and organ harvests; phage titering; and DNA sequencing work. Slides for histology were prepared in the Histology Department of Fletcher Allen Health Care (FAHC), Burlington, Vermont, a hospital affiliated with the University of Vermont College of Medicine, and read at the University of Vermont.

- **Record storage**

  All toxicology records are stored in Dr. Krag's laboratory, room E310 Given Building at the University of Vermont. Surgical reports and anesthesia records are stored in The Office of Animal Care, Given Building, University of Vermont.

Pharmacology and drug disposition

- **Pharmacological effects and mechanisms of action in animals**

  We do not anticipate that there will be a direct pharmacological effect caused by in vivo screening with random peptide phage-displayed libraries. The screening process is not a "drug" per se, but rather, a method of identifying promising new cancer therapeutics. Data from studies involving the injection of phage into humans by Dr. Ochs' group and Slopek et al[10-16], as well as our own data presented here from studies in mice, suggest that the pharmacological effects of the IV injection of the peptide-phage particles themselves will be minimal. The desired effect of the injections is a non-pharmacological one: to elute from tumor biopsies peptide-phage which bear peptides that bind specifically to tumor tissue.

Arap et al (see Appendix) have performed RPL in vivo screening experiments in mice with tumors, and have identified peptides which home specifically to tumor tissue. (They have also used this technique to identify small peptides which home specifically to 15 different normal organs[8]. Also refer to Rajotte et al in Appendix.) When the tumor-homing peptides were conjugated to doxorubicin (a chemotherapeutic used commonly to treat metastatic breast cancer), human breast cancer tumors growing in mice were eliminated extremely effectively with greatly lowered doxorubicin toxicity and impressive survival results. The ultimate goal of performing similar in vivo screening in humans is to achieve the same result: to identify peptides which home specifically to human tumors, which, when conjugated to a cytotoxic drug, will allow much more tumor-specific and non-toxic treatment of metastatic cancer. Present treatments are often ineffective and dangerously toxic to normal tissue. The ability to create cytotoxic drugs which home specifically to tumors, using homing peptides identified by in vivo screening, has the potential of revolutionizing the treatment of metastatic cancer.

Therefore, while the screening process itself, as described in this IND application, is not anticipated to have pharmacological effects, the final drug product *resulting* from in vivo RPL screening will specifically destroy or inhibit the growth of tumor cells in cancer patients.

The mechanism of action of the in vivo screening process is affinity enrichment, similar to mechanisms used by the immune system for B-cell and T-cell amplification of antigen binding clones. Millions or even billions of different peptides, displayed on phage particles, are injected directly into the bloodstream, and allowed to circulate throughout the organism. Phage which bear peptides which bind specifically to molecular targets located on tumor cells, or on endothelial cells of blood vessels specifically supplying tumor, will bind to those targets. The tumor is biopsied, phage present non-specifically are washed away, and the specific binding phage are eluted, amplified, and subjected to DNA analysis to determine the amino acid sequence of the binding peptide. Such peptides are potential tumor-homing peptides, which may allow specific delivery of cytotoxic agents to tumors.

The mechanism of action of the final peptide-cytotoxic agent conjugate would be specific delivery to the tumor via the tumor-homing peptide moiety. Cell-killing or growth inhibition, presumably, would be effected by the same mechanism utililized by the cytotoxic agent alone.

- **Info on absorption, distribution, metabolism and excretion if known**

  Although we can't entirely exclude a renal mechanism for excretion; the evidence, based on IHC results indicating the presence of phage particles in spleen and the Kupffer cells of the liver, supports clearance through the monocyte phagocytic system (MPS). Evidence of phage in feces would indicate hepatic involvement in clearance. Yip et al found phage in feces by immunohistochemistry 72 hours after injection of phage displaying Fab fragments (see Appendix). Our laboratory titered feces 3 weeks following phage injection and found no evidence of phage.

Toxicology Integrated Summary

Four in vivo studies (Study I-IV) were designed and implemented to assess the toxicity of phage random peptide library (RPL) screening in a mouse model. The toxicity studies were designed to mimic as closely as possible the scheme that will be used in phase I clinical trials. A total of 31 mice were injected with 3 different preparations of peptide phage (naïve peptide-phage, peptide-phage amplified once from tumor (φ Amp1x), or phage amplified twice from tumor (φ Amp2x)), and were monitored daily for three days or three weeks after phage injection for signs of toxicity. The FDA has suggested that 3 day and 3 week timepoints for organ harvest would allow us to evaluate both acute and chronic toxicity of peptide-phage injections. (See individual study sections for details.) At the end point of each study, 10 organs were harvested from each mouse and subjected to three analyses: hematoxylin & eosin staining (H&E) to assess pathology; immunohistochemistry (IHC) to look for the presence of phage particles (or at least intact phage coat proteins), which are not necessarily infective; and phage titering to determine the number of infective phage remaining. A brief description of each study follows. Complete details for each study may be found within each study section.

- Study I was designed to assess toxicity in mice, either 3 days or 3 weeks, following a single IV injection of naïve peptide-phage. This study will evaluate whether any toxicity will result from IV injection of phage particles. The number of each peptide present at this stage (approximately 20 picograms) is so small that any toxicity which might result would most certainly be caused by the phage particles alone. (Refer to Diagram of Study Design: Study I in Study I section.)

- Study II was designed to assess toxicity in mice following a single injection of phage amplified from tumor. This study will evaluate whether any toxicity will result from peptide-phage particles enriched for tumor binding. Specifically, IV injection of two types of peptide-phage will be evaluated: peptide-phage amplified from an in vivo tumor once; and peptide-phage passaged through in vivo tumors twice. This study will determine whether the peptides displayed on phage which have been passaged/enriched through an intact organism and its tumors, can cause toxicity not caused by the phage particles alone. The study will attempt to answer the following question: will peptide-phage amplified from host tissue be enriched for peptides that bind to normal host tissue in such a manner to cause toxicity? (Refer to Diagram of Study Design: Study II in Study II section.)

- Study III was designed to study toxicity in mice following three sequential IV injections of three different peptide-phage preparations: naïve peptide-phage library, peptide-phage amplified from a tumor in another mouse (φ Amp1x) and φ Amp1x passaged again through a second tumor (φ Amp2x). (Refer to Diagram of Study Design: Study III in Study III section.)

- Study IV was designed to study toxicity in tumor bearing mice following three sequential IV injections of three different phage preparations in the same mouse: (1)naïve library, (2)phage amplified from a tumor excised from the same animal (φ Amp1x) after injection of naïve peptide-phage library, and (3)phage amplified from a second tumor excised from the same animal (φ Amp2x) after injection of φ Amp1x. Each injection occurred on separate days, in the order listed, followed 10 minutes later by excision of tumor. (Refer to Diagram of Study Design: Study IV in Study IV

section.) The purpose of Study IV is to carry out in vivo screening in animals using a protocol which is nearly identical to the planned clinical protocol. Study IV examines the toxicity of the complete in vivo screening clinical protocol and also begins to examine efficacy, by determining whether the procedure can enrich for certain phage-displayed peptides, some of which may bind specifically to tumor tissue.

Three strains of mice (acquired from Jackson Labs in Bar Harbor, Maine) were used for the four toxicity studies described above: FVB, BalbC, and MRL/MpJ-fas$_{LPR}$(MRL). The FVB and BalbC mice are normal strains that have intact immune systems. However, the MRL mice develop massive lymph node enlargement, or lymphoproliferative disease, beginning around 8 weeks of age. Because their lymph nodes become markedly enlarged (tumors) these mice were chosen to provide us with tumors targets for our in vivo screening. While these mice have the advantage of producing tumors for screening, they have the disadvantage of dying rather early (and somewhat unpredictably, 3-5 months in our experience) compared to other strains of mice. Therefore, it was difficult to maintain a group of MRL mice that had enough tumors for our studies yet were healthy enough to survive 3 surgeries and live to the 3-week endpoint (as in Study IV).

Overall findings: (Please refer to individual Study sections for complete detail.)

- **Survival**: Of the 31 phage-injected mice in our studies, all but one survived to the endpoint. One of three surgical mice (#3), died while under general anesthesia following the removal of tumor during the second surgery. (Refer to the Appendix for Survival Surgery Protocol.) Note that there were 32 planned phage-injected mice. However, one of these (see Study II) died in a restraint while preparing the animal for injection.

- **Gross appearance**: All non-surgical mice, except for the one that died in the restraint, (n=28) appeared normal for the study duration as observed by activity level, appearance of coat, and posture. The mice that had surgeries to remove tumors (n=3) were somewhat less active the first day or two following each surgery. As discussed above, surgical mouse #3 died during the second surgery, therefore there are limited observations for this animal. (Refer to Study IV for more information.) Additionally, the surgical mice chewed and pulled their sutures; Mouse #2 removed its sutures to the point of opening an incision. The incision was cleaned and antibiotic ointment applied daily until the wound healed. Surgical mice (#1 and #2), which were subjected to a complete phage screening, similar to the clinical protocol except with more extensive surgery and anesthesia, progressed well to the end of the study.

- **Weights**: Mice were weighed daily, with the exception of some weekend measurements that are noted in the study sections. While we have not performed a statistical analysis of the data, generally the mice appear to have either maintained or gained weight (apparently, compared to controls, consistent with normal growth) throughout the course of each study with the following exceptions. One of the two groups of mice in Study II (mice injected with φ Amp1x) dropped an average of 9.1% (n=7) of their body weight on the day following injection with phage, but recovered from this weight drop by the following day. The surgical mice (Study IV) dropped about 8% of their body weight in the days immediately following surgeries and stabilized. This is not surprising in light of the fact that they underwent general anesthesia 2-3 times, in a short period of time, and had large tumors removed that accounted for up to 3% of the their body weight over the course of 3 surgeries. For example, Surgery

Mouse #2 weighed 47.6 g on the first day of surgery, and had 3 tumors removed in 5 days weighing 600 mg, 403.6 mg, and 431.8 mg respectively.

- **Phage titers**: Phage titers were performed to determine the number of infective phage present in tissues. On the day of harvest, tissues samples from each of 9 organs and in some cases blood, were collected. The tissues were weighed prior to being homogenized and incubated with *E. coli* for titering. (The titering procedure is described in detail in the Appendix.)

One group of mice tissues (in Study II) were titered at three days. Of these, all blood and tissues were positive for infective phage except for negative blood and liver of mouse #3, and negative spleen of mouse #2. Three weeks following phage injection, regardless of preparation (naïve, φ Amp1x, or φ Amp2x) all tissues were negative for infective phage in all non-surgical mice.

Of the two surgical mice that survived to the 3-week endpoint, one was injected with phage immediately before euthanasia for reasons detailed in Study IV, therefore the tissues were, as expected, positive for phage. Infective phage were found in all but the tumor tissue of the remaining surgery mouse (#2) at 3 weeks, although less than the amount of phage detected in tissues titered at 3 days.

Summary of Phage Titer Results[1]

Study	3 Day Harvest	3 Week Harvest
I	ND[2]	-
II	+[3]	-
III	ND	-
IV	ND	+[4]

[1] +/- denotes presence/absence of infective phage
[2] Not done
[3] All tissues positive except for: mouse #3 blood and liver, and mouse #2 spleen
[4] All tissues of surgical mouse #2 positive for phage except tumor

- **IHC**: Slides for IHC were prepared by the Histology Department at FAHC according to their protocol (see Appendix) using rabbit anti-M13 primary antibody (Sigma B7786, lot 038H4885) at a concentration of 0.73 µg/ml, a 1:10,000 dilution of the stock provided by our laboratory. Most tissues were negative for phage particles at three weeks. See table below.

Summary of IHC Results[1]

Study	3 Day Harvest	3 Week Harvest
I	-/+[2] (n=4)	- (n=4)
II φ Amp1x	- (n=3)	- (n=4)
φ Amp2x	-/+[3] (n=4)	-/+[5] (n=4)
III	ND[4]	- (n=6)
IV	ND[4]	-/+[6] (n=2)

[1]Staining is graded using a -to +++ scale for immunoreactivity.
[2]Spleens had trace/+ staining. All other tissues were negative.
[3]Livers of 3 out of 4 mice had + staining.
Spleens of 2 out of 4 mice had +/++ staining.
One lymph node had ++ staining.
One kidney had +/++ staining.
All other tissues were negative.
[4]Not done, by design.
[5]One liver and one lymph node had + staining. All other tissues were negative.
[6]All tissues negative for mouse #2.
+/++ staining in all mouse #1 tissues following injection with peptide-phage. See Study IV for details.

- **H&E:** All tissues in all studies were found to be of normal histology for the given strain except for 3 out of 4 FVB livers in Study II. The project pathologist found some histological differences inherent to the strain of mouse after looking at control animals. Specifically, MRL mice typically have: (1)enlarged lymph nodes, (2)enlarged spleens with markedly expanded white pulp with lymphoproliferative disorder, and (3)glomerulonephritis in the kidney. BalbCs also had a strain specific pathology in that they typically have steatosis of the liver. This was substantiated by looking at a control (no injections of any kind) BalbC liver. Therefore, we have qualified our pathology results by referring to data as "normal for the given strain" throughout the body of this document. Additionally, it is normal for mice in general to have spleens with extramedullary hematopoiesis.

Summary of H&E Results[1]

Study	3 Day Harvest	3 Week Harvest
I	NL (n=5)	NL (n=5)
II φ Amp1x	NL (n=3)	NL (n=4)
φ Amp2x	2 livers with hepatitis, remaining tissues NL (n=4)	1 liver with hepatitis, remaining tissues NL (n=4)
III	ND[2]	NL (n=6)
IV	ND[2]	NL (n=2)

[1]NL denotes normal histology for the strain
[2]Not done, by design.

The data summarized above accurately reflects the animal toxicology data from the completed studies.

SIGNATURE OF INDIVIDUALS WHO EVALUATED ANIMAL SAFETY DATA

David Krag

6/8/00

Date

Donald Weaver

6/8/00

Date

Study I

Toxicity Study in Mice Following One Injection of Naïve Library Peptide-Phage

Diagram of Study Design: Study I

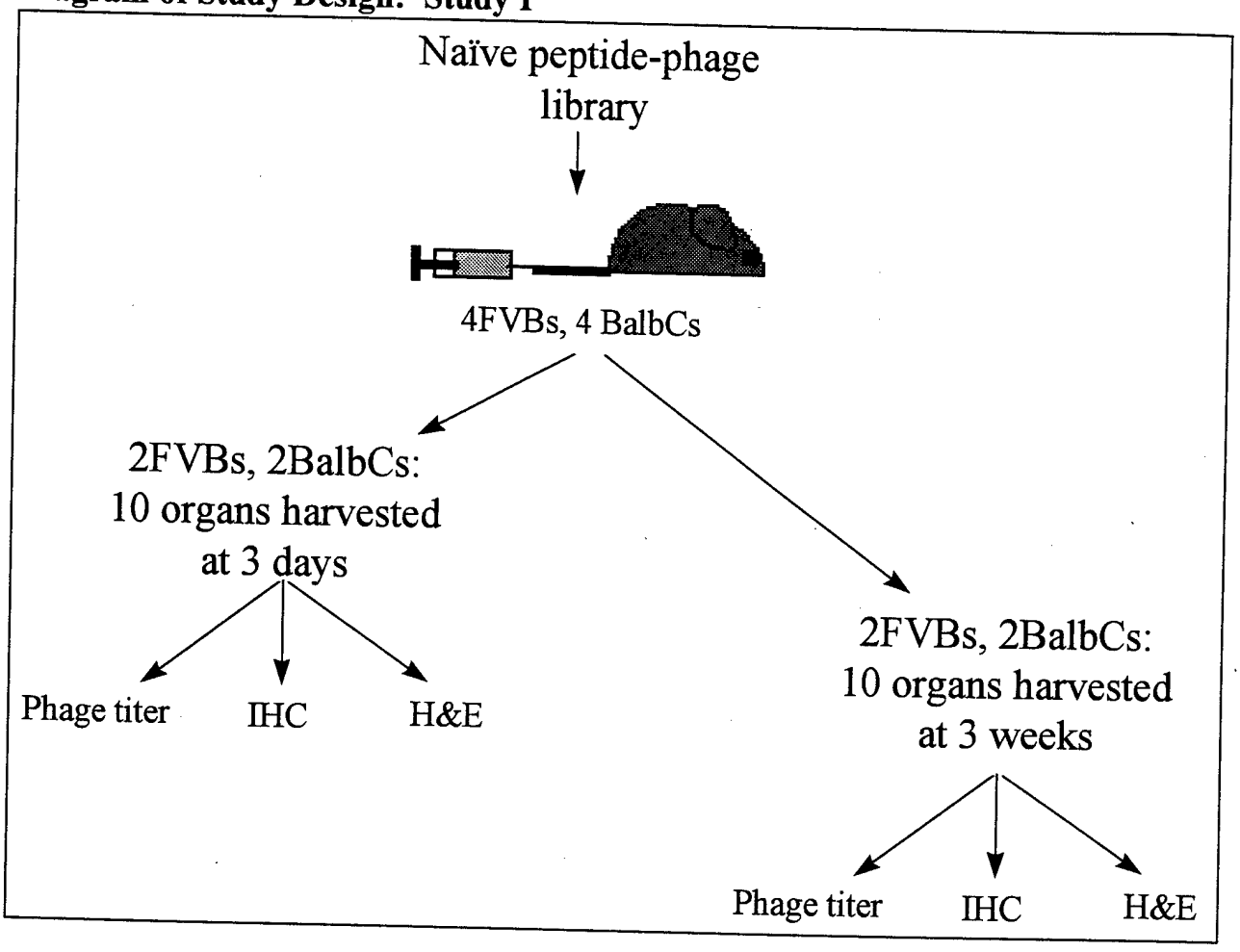


Table of Study Design: Study I

Mouse	Strain	Peptide-Phage Injection	Organ Harvest at 3 Days	Organ Harvest at 3 Weeks
1	FVB	+	X	
2	FVB	+	X	
3	FVB(control)	-	X	
4	BalbC	+	X	
5	BalbC	+	X	
6	FVB	+		X
7	FVB	+		X
8	FVB(control)	-		X
9	BalbC	+		X
10	BalbC	+		X

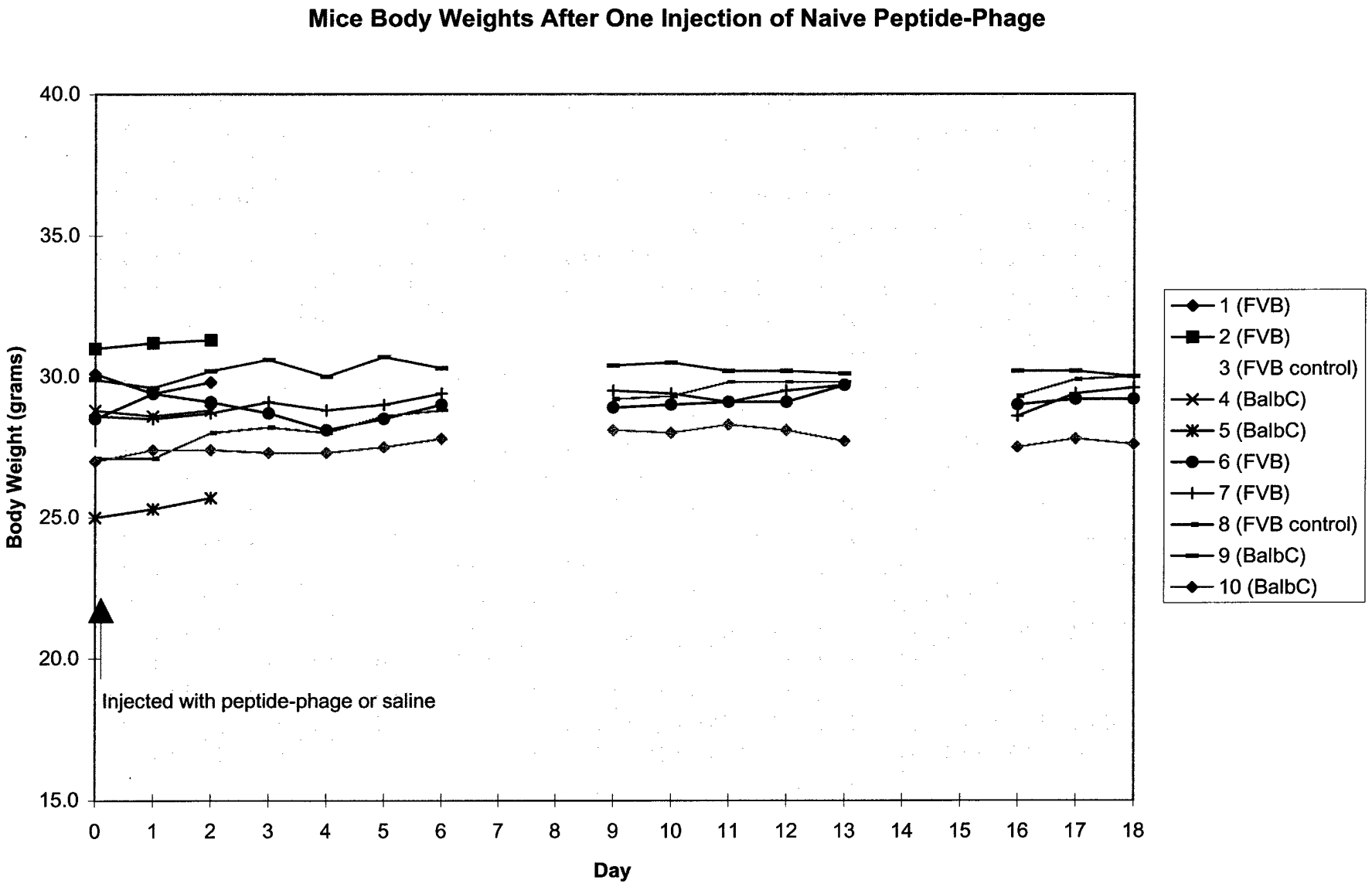
Methods: Study I

- Eight mice (4 FVBs, 4 BalbCs) were injected IV via the tail vein on 8/9/99 with a *single dose of naïve library phage*. Two additional mice (FVB) were injected with saline IV via the tail to serve as controls.
- Mice were monitored daily after peptide-phage injection until organ harvesting. During the monitoring period, mice were weighed and observed for signs of toxicity.
- Two mice of each strain were euthanized for organ harvest at 3 days (to assess acute toxicity). The remaining 2 mice of each strain were euthanized for organ harvest at 3 weeks (to assess chronic toxicity). Immediately following euthanasia, samples from ten organs (brain, diaphragm, heart, kidney, lung, bone marrow, lymph node, spleen, testis/uterus, and liver) were harvested, placed in buffered formalin, and sent to FAHC Histology for processing, H&E staining, and IHC staining. All slides were subsequently read by the project pathologist. Note that "gonad" is used throughout the body of this document to indicate testis or uterus.
- The same tissues were also collected (all but marrow, due to sample size limitations) and processed for phage titering, future PCR and confocal analysis. Blood was also collected and titered for phage at this time.

Summary of Results: Study I

- **Survival:** All mice survived to end point.
- **Gross appearance:** Activity, behavior, and appearance were observed to be normal in all mice for the duration of the study.
- **Weights:** All mouse weights appeared to be stable throughout the study. Measurements were not collected for weekend timepoints.
- **Phage Titers:** All tissue and blood titers for infective phage were negative at three weeks. The three day results were not obtained due to experimental error.
- **IHC:** Most tissues were negative for immunoreactivity, indicating the absence of phage particles. The only tissue positive for phage staining were the spleens of mice euthanized three days after peptide-phage injection.
- **H&E:** All tissues, both those harvested 3 days and 3 weeks following injection of naïve peptide phage, were determined to have normal histology.

Study I


Mice Body Weights After One Injection of Naive Peptide-Phage
Body Weight (grams)
40.0
35.0
30.0
25.0
20.0
15.0
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
Day
Injected with peptide-phage or saline
1 (FVB)
2 (FVB)
3 (FVB control)
4 (BalbC)
5 (BalbC)
6 (FVB)
7 (FVB)
8 (FVB control)
9 (BalbC)
10 (BalbC)

Study I

Body Weights for Mice Following One Injection of Naïve Peptide-Phage or Saline											
Strain		FVB	FVB	FVB	BalbC	BalbC	FVB	FVB	FVB	BalbC	BalbC
Phage Injected		08/09/99	08/09/99		08/09/99	08/09/99	08/09/99	08/09/99		08/09/99	08/09/99
Saline Injected				08/09/99					08/09/99		
		Mouse #									
Date	**Day**	1 (FVB)	2 (FVB)	3 (FVB control)	4 (BalbC)	5 (BalbC)	6 (FVB)	7 (FVB)	8 (FVB control)	9 (BalbC)	10 (BalbC
08/09/99	0	30.1	31.0	27.3	28.8	25.0	28.5	28.6	27.1	29.9	27.0
08/10/99	1	29.4	31.2	27.4	28.6	25.3	29.4	28.5	27.1	29.6	27.4
08/11/99	2	29.8	31.3	27.6	28.8	25.7	29.1	28.7	28.0	30.2	27.4
08/12/99	3						28.7	29.1	28.2	30.6	27.3
08/13/99	4						28.1	28.8	28.0	30.0	27.3
08/14/99	5						28.5	29.0	28.6	30.7	27.5
08/15/99	6						29.0	29.4	28.8	30.3	27.8
08/16/99	7						NA[1]	NA[1]	NA[1]	NA[1]	NA[1]
08/17/99	8						NA[1]	NA[1]	NA[1]	NA[1]	NA[1]
08/18/99	9						28.9	29.5	29.2	30.4	28.1
08/19/99	10						29.0	29.4	29.3	30.5	28.0
08/20/99	11						29.1	29.1	29.8	30.2	28.3
08/21/99	12						29.1	29.5	29.8	30.2	28.1
08/22/99	13						29.7	29.7	29.8	30.1	27.7
08/23/99	14						NA[1]	NA[1]	NA[1]	NA[1]	NA[1]
08/24/99	15						NA[1]	NA[1]	NA[1]	NA[1]	NA[1]
08/25/99	16						29.0	28.6	29.3	30.2	27.5
08/26/99	17						29.2	29.4	29.9	30.2	27.8
08/27/99	18						29.2	29.6	30.0	30.0	27.6
[1]Not available. Mice were not weighed on the weekends.											

Phage Titer Results: Study I[1]

3 Weeks After Naïve Peptide-Phage Injection

Mouse	6	7	8[2]	9	10
Tissue					
Blood	-	-	-	-	-
Liver	-	-	-	-	-
Spleen	-	-	-	-	-
Lymph node	-	-	-	-	-
Gonads	-	-	-	-	-
Heart	-	-	-	-	-
Lung	-	-	-	-	-
Kidney	-	-	-	-	-
Brain	-	-	-	-	-
Diaphragm	-	-	-	-	-

[1]+/- denotes the presence/absence of infective phage in the tissue.
[2]Control mouse injected with saline only.

IHC Results: Study I[1]

	3-Days After Naïve Peptide-Phage Injection					3 Weeks After Naïve Peptide-Phage Injection				
Mouse	**1**	**2**	**3[2]**	**4**	**5**	**6**	**7**	**8[2]**	**9**	**10**
Tissue										
Liver	-	-	-	-	-	-	-	-	-	-
Spleen	+[3]	+[4]	-	trace[5]	+	-	-	-	-	-
Lymph node	NA[6]	NA[6]	NA[6]	-	NA[6,7]	-	NA[6,7]	NA[6,7]	NA[6,7]	NA[6,7]
Gonads	-	-	-	-	-	-	-	NA[8]	-	-
Heart	-	-	-	-	-	-	-	-	-	-
Lung	-	-	-	-	-	-	-	-	-	-
Bone marrow	-	-	-	-	-	-	-	-	-	-
Kidney	-	-	-	-	-	-	-	-	-	-
Brain	-	-	-	-	-	-	-	NA[9]	-	-
Diaphragm	-	-	-	-	-	-	-	NA[10]	-	-

[1]Note a scale of - to +++ used to grade staining.
[2]Control mouse injected with saline only.
[3]+ focal staining in macrophages, only 2 isolated foci, white pulp in germinal centers/lymphocytes and macrophages.
[4]+ staining in lymphocytes of white pulp.
[5]Trace staining of germinal centers of lymphocytes.
[6]Not available. No lymph node tissue obtained. Normal lymph node is very small and often difficult to identify during harvest.
[7]Not available, only pancreas on slide.
[8]Not available, no testes on slide, only spermatic cord with negative staining.
[9]Not available, no brain on slide, only salivary gland with negative staining.
[10]Not available, no diaphragm on slide, only fatty connective tissue with negative staining.

Study I IHC

3 Days After Peptide-Phage Injection						
Using Rabbit α-M13 at 1:10,000 dilution (Sigma cat #B7786, lot#038H4885)						
Scale used to grade staining is - to +++						
Mouse	Harvested	Strain	Phage Injection	Tissue	Comments	Sex
1	08-12-99	FVB	naïve 8-9-99	Brain	negative staining, no immunoreactivity	male
				Diaphragm	negative staining, no immunoreactivity	
				Heart	negative staining, no immunoreactivity	
				Kidney	negative staining, no immunoreactivity	
				Lung	negative staining, no immunoreactivity	
				Marrow	negative staining, no immunoreactivity, hemosiderin present.	
				L.Node	no nodal tissue found	
				Spleen	**+ focal staining in macrophages, only 2 isolated foci,**	
					white pulp in germinal centers/lymphocytes and macrophage	
				Testes	negative staining, no immunoreactivity	
				Liver	negative staining, no immunoreactivity	
2	08-12-99	FVB	naïve 8-9-99	Brain	negative staining, no immunoreactivity	male
				Diaphragm	negative staining, no immunoreactivity	
				Heart	negative staining, no immunoreactivity	
				Kidney	negative staining, no immunoreactivity	
				Lung	negative staining, no immunoreactivity	
				Marrow	negative staining, no immunoreactivity	
				L.Node	no nodal tissue found	
				Spleen	**+ staining in lymphocytes of white pulp**	
				Testes	negative staining, no immunoreactivity	
				Liver	negative staining, no immunoreactivity	
3	08-12-99	FVB	Saline	Brain	negative staining, no immunoreactivity	male
				Diaphragm	negative staining, no immunoreactivity	
				Heart	negative staining, no immunoreactivity	
				Kidney	negative staining, no immunoreactivity	
				Lung	negative staining, no immunoreactivity	
				Marrow	negative staining, no immunoreactivity	
				L.Node	no nodal tissue found, fat only, neg staining	
				Spleen	negative staining, no immunoreactivity	
				Testes	negative staining, no immunoreactivity	
				Liver	negative staining, no immunoreactivity	
4	08-12-99	BalbC	naïve 8-9-99	Brain	negative staining, no immunoreactivity	male
				Diaphragm	negative staining, no immunoreactivity	
				Heart	negative staining, no immunoreactivity	
				Kidney	negative staining, no immunoreactivity	
				Lung	negative staining, no immunoreactivity	
				Marrow	negative staining, no immunoreactivity	
				L.Node	negative staining, no immunoreactivity	
				Spleen	**trace staining of germinal centers of lymphocytes**	
				Testes	negative staining, no immunoreactivity	
				Liver	negative staining, no immunoreactivity	
5	08-12-99	BalbC	naïve 8-9-99	Brain	negative staining, no immunoreactivity	male
				Diaphragm	negative staining, no immunoreactivity	
				Heart	negative staining, no immunoreactivity	
				Kidney	negative staining, no immunoreactivity	
				Lung	negative staining, no immunoreactivity	
				Marrow	negative staining, no immunoreactivity	
				L.Node	no node/pancreas--neg staining	
				Spleen	**+ staining in germinal centers of lymphocytes**	
				Testes	negative staining, no immunoreactivity	
				Liver	negative staining, no immunoreactivity	

Study I IHC

3 Weeks After Peptide-Phage Injection						
Using Rabbit α-M13 at 1:10,000 dilution (Sigma cat #B7786, lot#038H4885)						
Scale used to grade staining is - to +++			Phage			
Mouse	Harvested	Strain	Injection	Tissue	Comments	Sex
6	09-02-99	FVB	naïve 8-9-99	Brain	negative staining, no immunoreactivity	male
				Diaphragm	negative staining, no immunoreactivity	
				Heart	negative staining, no immunoreactivity	
				Kidney	negative staining, no immunoreactivity	
				Lung	negative staining, no immunoreactivity	
				Marrow	negative staining, no immunoreactivity	
				L.Node	negative staining, no immunoreactivity	
				Spleen	negative staining, no immunoreactivity	
				Testes	negative staining, no immunoreactivity	
				Liver	negative staining, no immunoreactivity	
7	09-02-99	FVB	naïve 8-9-99	Brain	negative staining, no immunoreactivity	male
				Diaphragm	negative staining, no immunoreactivity	
				Heart	negative staining, no immunoreactivity	
				Kidney	negative staining, no immunoreactivity	
				Lung	negative staining, no immunoreactivity	
				Marrow	negative staining, no immunoreactivity	
				L.Node	no node, pancreas on slide, neg staining	
				Spleen	negative staining, no immunoreactivity, a little hemosiderin	
				Testes	negative staining, no immunoreactivity	
				Liver	negative staining, no immunoreactivity	
8	09-02-99	FVB	Saline	Brain	no brain, salivary gland neg staining	male
				Diaphragm	no diaphragm, fatty connective tissue--neg staining	
				Heart	negative staining, no immunoreactivity	
				Kidney	negative staining, no immunoreactivity	
				Lung	negative staining, no immunoreactivity	
				Marrow	negative staining, no immunoreactivity	
				L.Node	no node, pancreas--negative staining	
				Spleen	negative staining, no immunoreactivity	
				Testes	no testes, spermatic cord--negative staining	
				Liver	negative staining, no immunoreactivity	
9	09-02-99	BalbC	naïve 8-9-99	Brain	negative staining, no immunoreactivity	male
				Diaphragm	negative staining, no immunoreactivity	
				Heart	negative staining, no immunoreactivity	
				Kidney	negative staining, no immunoreactivity	
				Lung	negative staining, no immunoreactivity	
				Marrow	negative staining, no immunoreactivity	
				L.Node	no node, pancreas--negative staining	
				Spleen	negative staining, no immunoreactivity	
				Testes	negative staining, no immunoreactivity	
				Liver	negative staining, no immunoreactivity	
10	09-02-99	BalbC	naïve 8-9-99	Brain	negative staining, no immunoreactivity	male
				Diaphragm	negative staining, no immunoreactivity	
				Heart	negative staining, no immunoreactivity	
				Kidney	negative staining, no immunoreactivity	
				Lung	negative staining, no immunoreactivity	
				Marrow	negative staining, no immunoreactivity	
				L.Node	no node, pancreas--negative staining	
				Spleen	negative staining, no immunoreactivity	
				Testes	negative staining, no immunoreactivity	
				Liver	negative staining, no immunoreactivity	

H&E Results: Study I

	3-Days After Naïve Peptide-Phage Injection					3 Weeks After Naïve Peptide-Phage Injection				
Mouse	**1**	**2**	**3[1]**	**4**	**5**	**6**	**7**	**8[1]**	**9**	**10**
Tissue										
Liver	NL[2]	NL	NL	NL	NL	NL	NL	NL	NL	NL
Spleen	NL	NL	NL	NL	NL	NL	NL	NL	NL	NL
Lymph node	NA[3]	NA[3]	NA[3]	NL	NA[3, 4]	NL	NA[3, 4]	NA[3, 4]	NA[3, 4]	NA[3, 4]
Gonads	NL	NL	NL	NL	NL	NL	NL	NA[8]	NL	NL
Heart	NL	NL	NL	NL	NL	NL	NL	NL	NL	NL
Lung	NL	NL	NL	NL	NL	NL	NL	NL	NL	NL
Bone marrow	NL	NA[5]	NL	NL	NL	NL	NA[5]	NL	NL	NL
Kidney	NL	NL	NL	NL	NL	NL	NL	NL	NL	NL
Brain	NL	NL	NL	NL	NL	NL	NL	NA[6]	NL	NL
Diaphragm	NL	NL	NL	NL	NL	NL	NL	NA[7]	NL	NL

[1]Control mouse injected with saline only.
[2]NL denotes normal histology for the strain. See explanation in Integrated Summary.
[3]Not available. No lymph node tissue obtained. Normal lymph node is very small and often difficult to identify during harvest.
[4]Not available. Only pancreas on slide.
[5]Not available. Insufficient sample obtained at time of harvest. Only enough obtained for IHC.
[6]Not available. No brain on slide, only salivary gland.
[7]Not available. Only fatty connective tissue found on slide.
[8]Not available. Only spermatic cord found on slide.

Study I H+E

3 Days After Peptide-Phage Injection							
Mouse	Harvested	Strain	Phage Injection	Tissue	Pathology	Comments	Sex
1	08-12-99	FVB	naïve 8-9-99	Brain	normal	minimal hypoxic neuronal change	male
				Diaphragm	normal	some chronic interstitial inflam. cells, some chronic myositis	
				Heart	normal		
				Kidney	normal	subtle increase in cellularity of glomeruli, little protein in tubules-not glassy	
				Lung	normal	with minimal pulmonary edema	
				Marrow	normal	megakeryocytes, pmns, monocytes	
				L.Node	not available	no nodal tissue obtained, looks like duodenum	
				Spleen	normal	extra medullary hematopoiesis, some stimulation of white pulp	
				Testes	normal	maturing spermatogenisis	
				Liver	normal		
2	08-12-99	FVB	naïve 8-9-99	Brain	normal		male
				Diaphragm	normal		
				Heart	normal		
				Kidney	normal	some protein in tubules	
				Lung	normal		
				Marrow	not available		
				L.Node	not available	fat obtained	
				Spleen	normal	extra medullary hematopoiesis, some stimulation of white pulp	
				Testes	normal	maturing spermatogenisis	
				Liver	normal		
3	08-12-99	FVB	Control	Brain	normal	minimal hypoxic neuronal change	male
				Diaphragm	normal		
				Heart	normal		
				Kidney	normal	subtle increase in cellularity of glomeruli, little protein in tubules-not glassy	
				Lung	normal	with minimal pulmonary edema	
				Marrow	normal	megakeryocytes, pmns, monocytes	
				L.Node	not available	fat on slide/ no nodal tissue obtained	
				Spleen	normal	extra medullary hematopoiesis, some stimulation of white pulp	
				Testes	normal	maturing spermatogenisis	
				Liver	normal		
4	08-12-99	BalbC	naïve 8-9-99	Brain	normal		male
				Diaphragm	normal	some xyphoid present	
				Heart	normal		
				Kidney	normal		
				Lung	normal		
				Marrow	normal		
				L.Node	normal		
				Spleen	normal		
				Testes	normal		
				Liver	normal	moderate macro steatosis	
5	08-12-99	BalbC	naïve 8-9-99	Brain	normal		male
				Diaphragm	normal		
				Heart	normal		
				Kidney	normal		
				Lung	normal	prominent terminal bronchioles	
				Marrow	normal		
				L.Node	not available	pancreas on slide	
				Spleen	normal		
				Testes	normal		
				Liver	normal	minimal macro steatosis	

Study I H+E

3 Weeks After Peptide-Phage Injection							
Mouse	Harvested	Strain	Phage Injection	Tissue	Pathology	Comments	Sex
6	09-02-99	FVB	naïve 8-9-99	Brain	normal		male
				Diaphragm	normal		
				Heart	normal		
				Kidney	normal		
				Lung	normal		
				Marrow	normal		
				L.Node	normal		
				Spleen	normal		
				Testes	normal		
				Liver	normal		
7	09-02-99	FVB	naïve 8-9-99	Brain	normal		male
				Diaphragm	normal		
				Heart	normal		
				Kidney	normal		
				Lung	normal		
				Marrow	not available		
				L.Node	not available	pancreas on slide	
				Spleen	normal		
				Testes	normal		
				Liver	normal	no steatosis observed	
8	09-02-99	FVB	Control	Brain	not available	salivary gland ?	male
				Diaphragm	not available	fatty connective tissue	
				Heart	normal		
				Kidney	normal		
				Lung	normal		
				Marrow	normal		
				L.Node	not available	pancreas on slide	
				Spleen	normal		
				Testes	not available	spermatic cord on slide	
				Liver	normal	no steatosis observed	
9	09-02-99	BalbC	naïve 8-9-99	Brain	normal		male
				Diaphragm	normal		
				Heart	normal		
				Kidney	normal	shrunken glomeruli	
				Lung	normal		
				Marrow	normal		
				L.Node	not available	pancreas on slide	
				Spleen	normal	extra medullary hematopoiesis	
				Testes	normal		
				Liver	normal	moderate steatosis always around zone 3 (central vein), questionable focal steato-hepatitis	
10	09-02-99	BalbC	naïve 8-9-99	Brain	normal		male
				Diaphragm	normal		
				Heart	normal		
				Kidney	normal		
				Lung	normal		
				Marrow	normal		
				L.Node	not available	pancreas on slide	
				Spleen	normal	extramedullary hematopoiesis	
				Testes	normal		
				Liver	normal		
Extra Control mouse to look at liver since three out of four Balb C's (all injected with phage) had steatosis.							
	12-15-99	BalbC-BYJ	Control	liver	normal	**moderate steatosis, ususally zones 2 and 3**	

Study II
Toxicity Study in Mice Following One Injection of Phage Amplified From Tumor

Diagram of Design: Study II

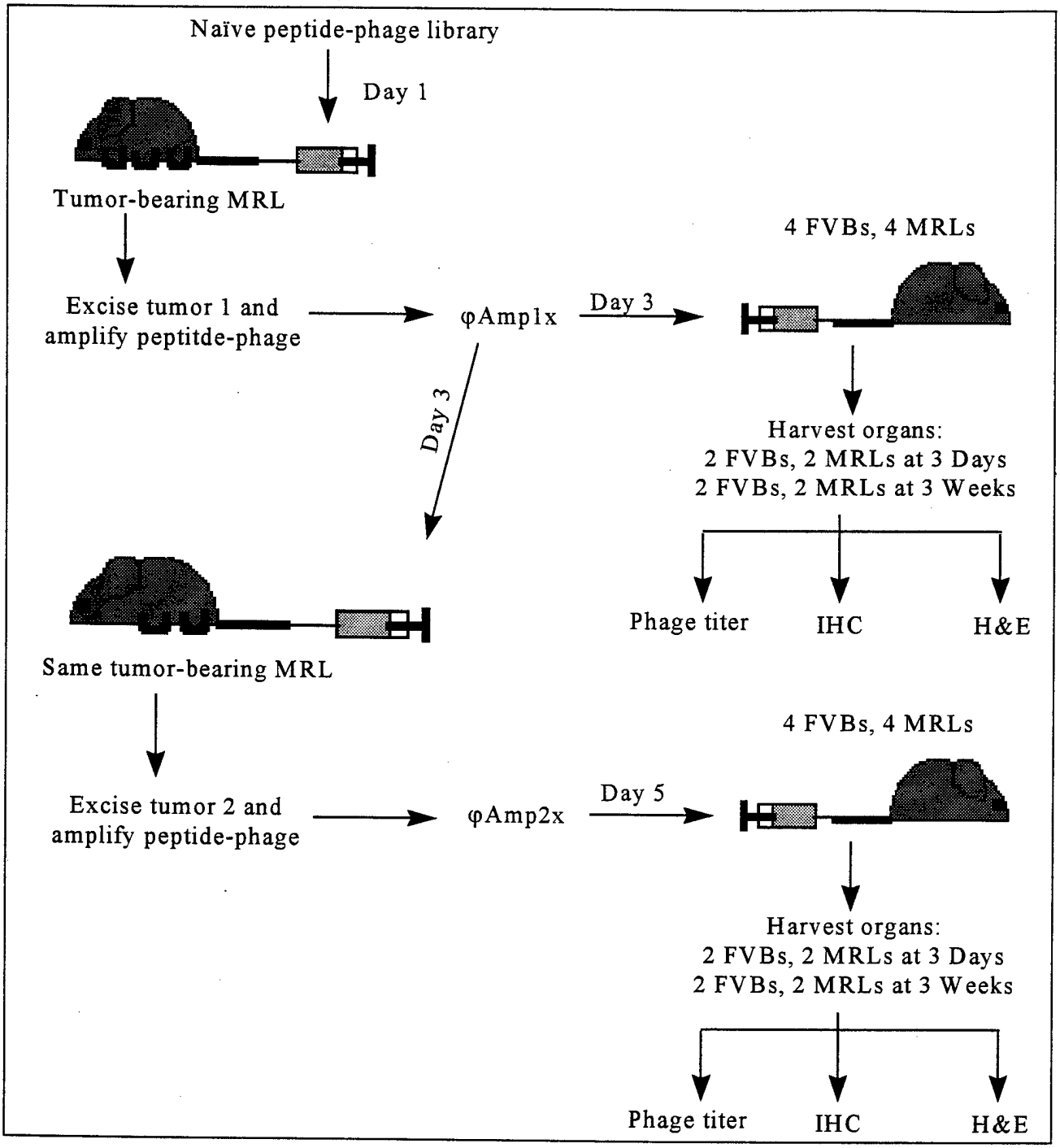


Table of Study Design: Study II

Mouse	Strain	φ Amp1x	Organ Harvest at 3 Days	Organ Harvest at 3 Weeks
1	FVB	+	X	
2	FVB	+	X	
3	MRL	+	X	
4	MRL	+	X	
5	FVB	+		X
6	FVB	+		X
7	MRL	+		X
8	MRL	+		X
Mouse	**Strain**	**φ Amp2x**	**Organ Harvest at 3 Days**	**Organ Harvest at 3 Weeks**
9	MRL	+	X	
10	MRL	+	X	
11	FVB	+	X	
12	FVB	+	X	
13	MRL	+		X
14	MRL	+		X
15	FVB	+		X
16	FVB	+		X

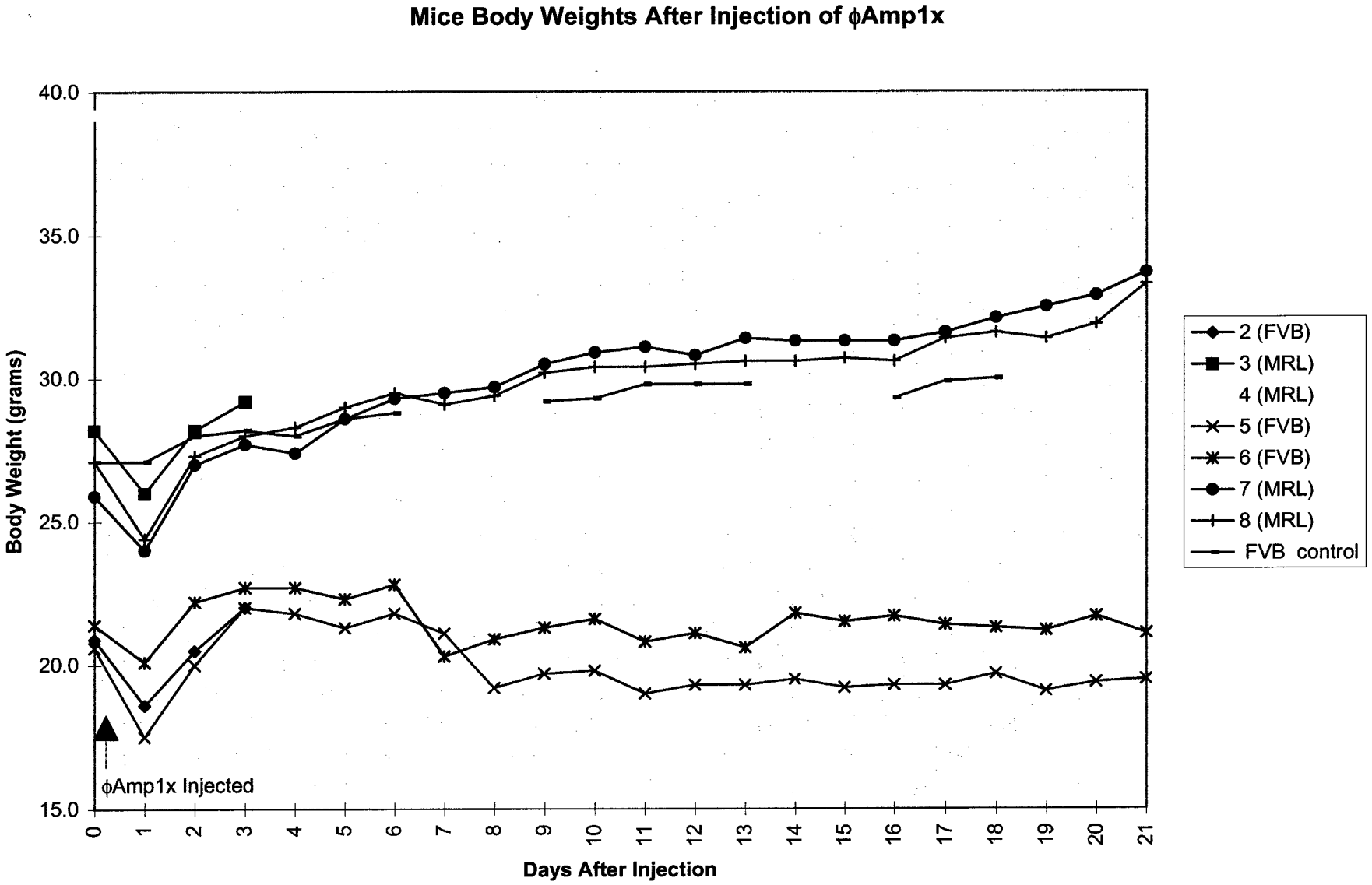
Methods: Study II

- On 2/2/00, seven mice (3 FVB, 4 MRL) were injected via the tail vein with 6.4×10^8 φAmp1x in a volume of 250 µl PBS. φAmp1x = peptide-phage amplified from tumor 1 excised from Survival Surgery Mouse #2 on 1/31/00. (See Study IV for details on survival surgery mice.) Note that 4 FVB mice were to be injected however, one died in the restraint prior to injection and there were no more mice of this strain on hand to replace the animal in the group.
- On 2/4/00, eight mice (4FVB, 4 MRL) were injected via the tail vein with 8.2×10^{10} φAmp2x in a volume of 245 µl PBS. φAmp2x = peptide-phage amplified from tumor 2 excised from Survival Surgery Mouse #2 on 2/2/00. (Refer to Study IV for details on survival surgery mice.)
- Mice were monitored daily after peptide-phage injection until organ harvesting. During the monitoring period, mice were weighed and observed for signs of toxicity.
- Two mice of each strain, from each group (φAmp1x and φAmp2x-injected mice) were euthanized for organ harvest at 3 days (to assess acute toxicity) or 3 weeks (to assess chronic toxicity). Immediately following euthanasia, samples from ten organs (brain, diaphragm, heart, kidney, lung, bone marrow, lymph node, spleen , gonads, and liver) were harvested, placed in buffered formalin and sent to FAHC Histology for processing, H&E staining, and IHC staining. All slides were subsequently read by the project pathologist.
- The same tissues were also collected (all but marrow, due to small sample size) and processed for phage titering, future PCR and confocal analysis. Blood was also collected and titered for phage at this time.

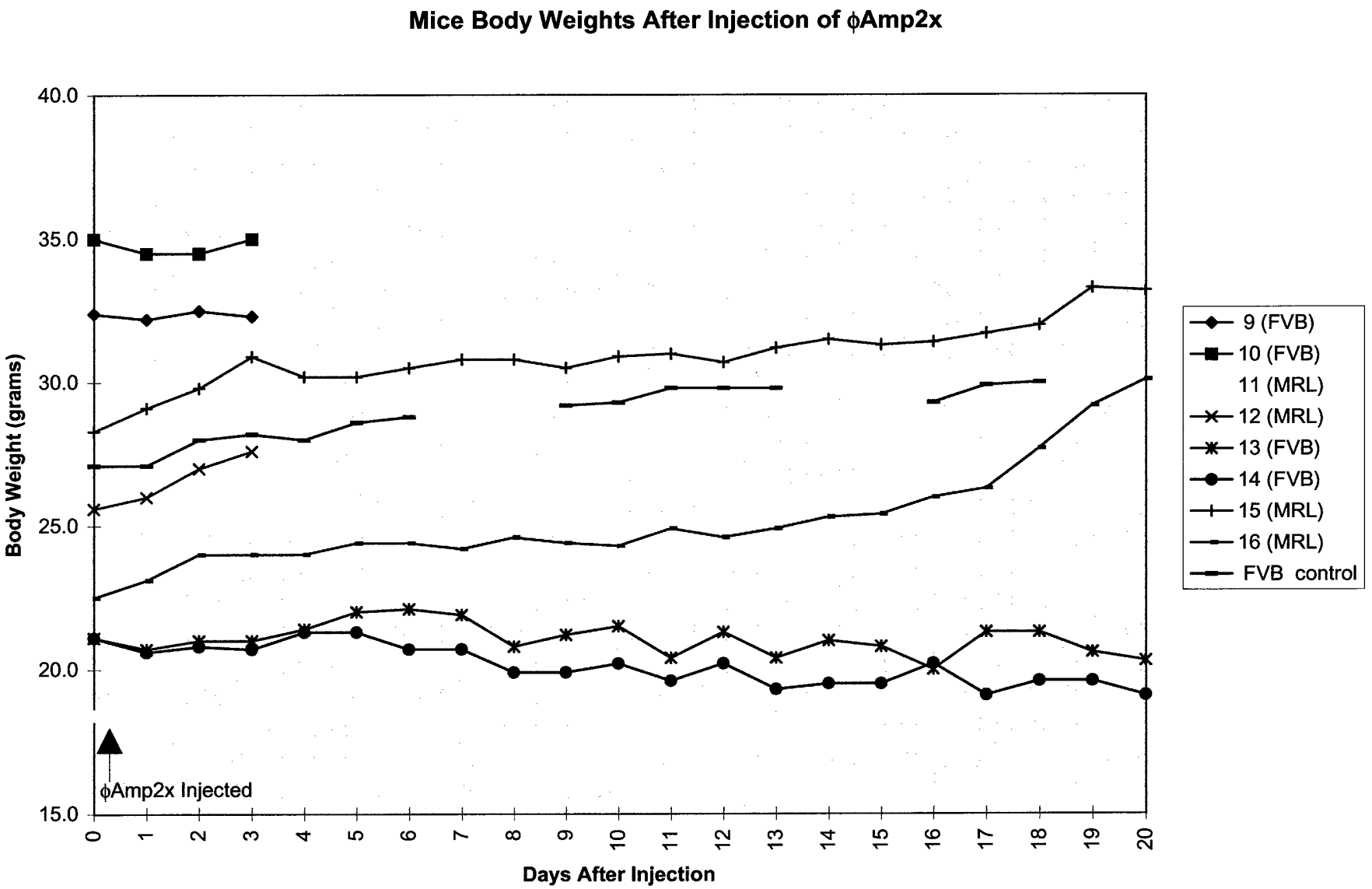
Summary of Results: Study II

- **Survival:** All mice survived to end point, except for one that died in the restraint prior to injection. This mouse was not replaced in the study due to a lack of available FVB mice.
- **Gross appearance:** Activity, behavior, and appearance were observed to be normal in all mice for the duration of the study.
- **Weights:** The mice injected with φ Amp1x dropped an average of 9.1% (n=7) of their body weight on day 1 following injection but had returned to baseline weights by day 2. The mice injected with φ Amp2x appear to have either maintained or gained weight throughout the remainder of the study.
- **Phage Titers:** Three days after phage injection there were infective phage present in all of the mice tissues except for the blood and liver of mouse #3, and the spleen of mouse #2 from φ Amp1x-injected mice. No infective phage were detected in any of the tissues collected three weeks after injection of either φ Amp1x or φ Amp2x.
- **IHC:** All tissues were negative for phage staining three days following injection of φ Amp1x. Most tissues from mice injected with φ Amp2x were negative at three days with the exceptions of: 3 livers, 2 spleens, 1 lymph node, and 1 kidney. All tissues three weeks following phage injection were negative for phage particles except for the liver of mouse #16, and a lymph node of mouse #15, both from the φ Amp2x-injected group.
- **H&E:** All tissues examined were normal for the strain with the exceptions of: (1) hepatitis findings in the livers of 3 FVB mice, and (2) one FVB liver with lymphoid aggregates. Sections of liver from mice with hepatitis were subsequently stained with Steiner Stain to rule out Helicobacter or Clostridium infection. No bacteria were identified. Refer to the tables for additional information.

Study II


Mice Body Weights After Injection of ϕAmp1x
Body Weight (grams)
40.0
35.0
30.0
25.0
20.0
15.0
ϕAmp1x Injected
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
Days After Injection
2 (FVB)
3 (MRL)
4 (MRL)
5 (FVB)
6 (FVB)
7 (MRL)
8 (MRL)
FVB control

Study II


Mice Body Weights After Injection of ϕAmp2x
Body Weight (grams)
40.0
35.0
30.0
25.0
20.0
15.0
ϕAmp2x Injected
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
Days After Injection
9 (FVB)
10 (FVB)
11 (MRL)
12 (MRL)
13 (FVB)
14 (FVB)
15 (MRL)
16 (MRL)
FVB control

Study II

Body Weights (grams) for Mice in Aim II Study											
		Mice Injected Once with ϕAmp 1x									
		(phage amplified from tumor excised from Survival Surgery Mouse #2 on 1/31/00)									
Strain		FVB	FVB	MRL	MRL	FVB	FVB	MRL	MRL		
Phage injected		02/02/00	02/02/00	02/02/00	02/02/00	02/02/00	02/02/00	02/02/00	02/02/00		
		died in									
		restraint									
Euthanized @			3 Days	3 Days	3 Days	3 Weeks	3 Weeks	3 Weeks	3 Weeks		
Date	Day		2 FVB	3 MRL	4 MRL	5 FVB	6 FVB	7 MRL	8 MRL	FVB control[1]	
01/31/00	-2									27.1	
02/01/00	-1									27.1	
02/02/00	0		20.9	28.2	39.2	20.6	21.4	25.9	27.1	28	
02/03/00	1		18.6	26.0	36.7	17.5	20.1	24.0	24.4	28.2	
02/04/00	2		20.5	28.2	39.4	20.0	22.2	27.0	27.3	28	
02/05/00	3		22.0	29.2	39.4	22.0	22.7	27.7	28.0	28.6	
02/06/00	4					21.8	22.7	27.4	28.3	28.8	
02/07/00	5					21.3	22.3	28.6	29.0	NA[2]	
02/08/00	6					21.8	22.8	29.3	29.5	NA[2]	
02/09/00	7					21.1	20.3	29.5	29.1	29.2	
02/10/00	8					19.2	20.9	29.7	29.4	29.3	
02/11/00	9					19.7	21.3	30.5	30.2	29.8	
02/12/00	10					19.8	21.6	30.9	30.4	29.8	
02/13/00	11					19.0	20.8	31.1	30.4	29.8	
02/14/00	12					19.3	21.1	30.8	30.5	NA[2]	
02/15/00	13					19.3	20.6	31.4	30.6	NA[2]	
02/16/00	14					19.5	21.8	31.3	30.6	29.3	
02/17/00	15					19.2	21.5	31.3	30.7	29.9	
02/18/00	16					19.3	21.7	31.3	30.6	30	
02/19/00	17					19.3	21.4	31.6	31.4		
02/20/00	18					19.7	21.3	32.1	31.6		
02/21/00	19					19.1	21.2	32.5	31.4		
02/22/00	20					19.4	21.7	32.9	31.9		
02/23/00	21					19.5	21.1	33.7	33.3		
02/24/00	22										

[1]Note that this control mouse was from Study I .

[2]Not available, mouse was not weighed on the weekends.

Study II

Body Weights (grams) for Mice in Aim II Study											
		Mice Injected Once with ϕAmp 2x									
		(amplified from tumor excised from Survival Surgery Mouse #2 on 02/02/00)									
Strain		FVB	FVB	MRL	MRL	FVB	FVB	MRL	MRL		
Phage injected		02/04/00	02/04/00	02/04/00	02/04/00	02/04/00	02/04/00	02/04/00	02/04/00		
Euthanized @		3 Days	3 Days	3 Days	3 Days	3 Weeks	3 Weeks	3 Weeks	3 Weeks		
		Mouse ID									
Date	**Day**	9 FVB	10 FVB	11MRL	12 MRL	13 FVB	14 FVB	15 MRL	16 MRL	FVB control[1]	
02/04/00	0	32.4	35.0	18.4	25.6	21.1	21.1	28.3	22.5	27.1	
02/05/00	1	32.2	34.5	18.0	26.0	20.7	20.6	29.1	23.1	27.1	
02/06/00	2	32.5	34.5	18.5	27.0	21.0	20.8	29.8	24.0	28	
02/07/00	3	32.3	35.0	18.3	27.6	21.0	20.7	30.9	24.0	28.2	
02/08/00	4					21.4	21.3	30.2	24.0	28	
02/09/00	5					22.0	21.3	30.2	24.4	28.6	
02/10/00	6					22.1	20.7	30.5	24.4	28.8	
02/11/00	7					21.9	20.7	30.8	24.2	NA[2]	
02/12/00	8					20.8	19.9	30.8	24.6	NA[2]	
02/13/00	9					21.2	19.9	30.5	24.4	29.2	
02/14/00	10					21.5	20.2	30.9	24.3	29.3	
02/15/00	11					20.4	19.6	31.0	24.9	29.8	
02/16/00	12					21.3	20.2	30.7	24.6	29.8	
02/17/00	13					20.4	19.3	31.2	24.9	29.8	
02/18/00	14					21.0	19.5	31.5	25.3	NA[2]	
02/19/00	15					20.8	19.5	31.3	25.4	NA[2]	
02/20/00	16					20.0	20.2	31.4	26.0	29.3	
02/21/00	17					21.3	19.1	31.7	26.3	29.9	
02/22/00	18					21.3	19.6	32.0	27.7	30	
02/23/00	19					20.6	19.6	33.3	29.2		
02/24/00	20					20.3	19.1	33.2	30.1		

[1]Note that this control mouse was from Study I .

[2]Not available, mouse was not weighed on the weekends.

Phage Titer Results: Study II[1]

	3 Days After φ Amp1x Injection				3 Weeks After φ Amp1x Injection			
Mouse 1		**2**	**3**	**4**	**5**	**6**	**7**	**8**
Tissue								
Blood	NA[2]	+	-	+	-	-	-	-
Liver	NA	+	-	+	-	-	-	-
Spleen	NA	-	+	+	-	-	-	-
Lymph node	NA	+	+	+	-	-	-	-
Gonads	NA	+	+	+	-	-	-	-
Heart	NA	+	+	+	-	-	-	-
Lung	NA	+	+	+	-	-	-	-
Kidney	NA	+	+	+	-	-	-	-
Brain	NA	+	+	+	-	-	-	-
Diaphragm	NA	+	+	+	-	-	-	-

	3 Days After φ Amp1x Injection				3 Weeks After φ Amp1x Injection			
Mouse 9		**10**	**11**	**12**	**13**	**14**	**15**	**16**
Tissue								
Blood	+	+	+	+	-	-	-	-
Liver	+	+	+	+	-	-	-	-
Spleen	+	+	+	+	-	-	-	-
Lymph node	+	+	+	+	-	-	-	-
Gonads	+	+	+	+	-	-	-	-
Heart	+	+	+	+	-	-	-	-
Lung	+	+	+	+	-	-	-	-
Kidney	+	+	+	+	-	-	-	-
Brain	+	+	+	+	-	-	-	-
Diaphragm	+	+	+	+	-	-	-	-

[1]+/- denotes the presence/absence of infective phage in the tissue.
[2]Not available, mouse died in restraint prior to injection.

IHC Results: Study II[1]

	3 Days After φ Amp1x Injection				3 Weeks After φ Amp1x Injection			
Mouse	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Tissue								
Liver	NA[2]	-	-	-	-	-	-	-
Spleen	NA	-	-	-	-	-	-	-
Lymph node	NA	-	-	-	-	-	-	-
Gonads	NA	-	-	-	-	-	-	-
Heart	NA	-	-	-	-	-	-	-
Lung	NA	-	-	-	-	-	-	-
Bone marrow	NA	-	NA[3]	-	-	-	-	NA[3]
Kidney	NA	-	-	-	-	-	-	-
Brain	NA	-	-	-	-	-	-	-
Diaphragm	NA	-	-	-	-	-	-	-

	3 Days After φ Amp1x Injection				3 Weeks After φ Amp1x Injection			
Mouse	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**
Tissue								
Liver	+[4]	+[4]	+[4]	-	-	-	-	+[12]
Spleen	-	+[6]	++[8]	-	-	-	-	-
Lymph node	-	-	++[9]	-	-	-	+[11]	-
Gonads	-	-	-	-	-	NA[13]	-	-
Heart	-	-	+[10]	-	-	-	-	-
Lung	-	-	-	-	-	-	-	-
Bone marrow	-	-	-	-	-	-	-	-
Kidney	+[5]	+/++[7]	-	-	-	-	-	-
Brain	-	-	-	-	-	-	-	-
Diaphragm	-	-	-	-	-	-	-	-

[1]Note a scale of - to +++ used to grade staining.
[2]Not available, mouse died in restraint prior to injection.
[3]Not available, inadequate sample size.
[4]Diffuse + staining in Kupffer cells
[5]Focal staining in histiocytes (?artifact)
[6]+ staining in germinal center lymphocytes and 1+ in mantel zone
[7]+/++ staining in glomeruli
[8] ++staining in germinal centers, patchy ++ staining in mantel zone and sinuses
[9]Focal ++ staining within germinal center folicles
[10]Focal endothelial staining
[11]Focal staining in interfolicular lymphocytes and macrophages
[12]Focal weak staining in Kupffer cells
[13]Not available, uterus was not harvested due to technical error.

Study II IHC

3 Days After Injection with Phage Amplified Once (from tumor 1 excised from Survival Surgery Mouse #2 on 1-31-00)						
Using Rabbit α-M13 at 1:10,000 dilution (Sigma cat #B7786, lot #038H4885)						
Scale used to grade staining is - to +++						
			Phage			
Mouse	Harvested	Strain	Injection	Tissue	Comments	Sex
1	02/05/00	FVB	ϕ Amp1x 02/02/00	Brain	Animal died in restraint during tail vein injection	
				Diaphragm		
				Heart		
				Kidney		
				Lung		
				Marrow		
				L.Node/tumor		
				Spleen		
				Uterus		
				Liver		
2	02/05/00	FVB	ϕ Amp1x 02/02/00	Brain	negative staining, no immunoreactivity	female
				Diaphragm	negative staining, no immunoreactivity	
				Heart	negative staining, no immunoreactivity	
				Kidney	negative staining, no immunoreactivity	
				Lung	negative staining, no immunoreactivity	
				Marrow	negative staining, no immunoreactivity	
				L.Node/tumor	negative staining, no immunoreactivity	
				Spleen	mild hemosiderin in histiocytes, diffusely distributed	
				Uterus	negative staining, no immunoreactivity	
				Liver	negative staining, no immunoreactivity	
3	02/05/00	MRL/MpJ-fas$_{LPR}$	ϕ Amp1x 02/02/00	Brain	negative staining, no immunoreactivity	male
				Diaphragm	negative staining, no immunoreactivity	
				Heart	negative staining, no immunoreactivity	
				Kidney	negative staining, no immunoreactivity	
				Lung	negative staining, no immunoreactivity	
				Marrow	no marrow found on slide	
				L.Node/tumor	negative staining, no immunoreactivity	
				Spleen	negative staining, no immunoreactivity	
				Uterus	negative staining, no immunoreactivity	
				Liver	negative staining, no immunoreactivity	
4	02/05/00	MRL/MpJ-fas$_{LPR}$	ϕ Amp1x 02/02/00	Brain	negative staining, no immunoreactivity	female
				Diaphragm	negative staining, no immunoreactivity	
				Heart	negative staining, no immunoreactivity	
				Kidney	negative staining, no immunoreactivity	
				Lung	negative staining, no immunoreactivity	
				Marrow	negative staining, no immunoreactivity	
				L.Node/tumor	negative staining, no immunoreactivity	
				Spleen	negative staining, no immunoreactivity	
				Uterus	negative staining, no immunoreactivity	
				Liver	negative staining, no immunoreactivity	
Mouse injected with phage on day of harvest to serve as positive control.						
	2/5/00	MRL/MpJ-fas$_{LPR}$	2/5/00	Blood	staining around erythrocyte membranes	
				Lung	interstitium staining, RBC's and endothelium stained	
				Spleen	hematopoiesis, sinusoidal staining	

Study II IHC

3 Weeks After Injection with Phage Amplified Once (from tumor 1 excised from Survival Surgery Mouse #2 on 1-31-00)						
Using Rabbit α-M13 at 1:10,000 dilution (Sigma cat #B7786, lot #038H4885)						
Scale used to grade staining is - to +++						
Mouse	Harvested	Strain	Phage Injection	Tissue	Comments	Sex
5	2/23/00	FVB	ϕ Amp1x 02/02/00	Brain	negative staining, no immunoreactivity	female
				Diaphragm	negative staining, no immunoreactivity	
				Heart	negative staining, no immunoreactivity	
				Kidney	negative staining, no immunoreactivity	
				Lung	negative staining, no immunoreactivity	
				Marrow	negative staining, no immunoreactivity	
				L.Node	negative staining, no immunoreactivity	
				Spleen	moderate hemosiderin in histiocytes, diffusely distributed	
				Uterus	negative staining, no immunoreactivity	
				Liver	negative staining, no immunoreactivity	
6	2/23/00	FVB	ϕ Amp1x 02/02/00	Brain	negative staining, no immunoreactivity	female
				Diaphragm	negative staining, no immunoreactivity	
				Heart	negative staining, no immunoreactivity	
				Kidney	negative staining, no immunoreactivity	
				Lung	negative staining, no immunoreactivity	
				Marrow	negative staining, no immunoreactivity	
				L.Node	negative staining, no immunoreactivity	
				Spleen	moderate hemosiderin in histiocytes, diffusely distributed	
				Uterus	negative staining, no immunoreactivity	
				Liver	negative staining, no immunoreactivity	
7	2/23/00	MRL/MpJ-fas_{LPR}	ϕ Amp1x 02/02/00	Brain	negative staining, no immunoreactivity	male
				Diaphragm	negative staining, no immunoreactivity	
				Heart	negative staining, no immunoreactivity	
				Kidney	negative staining, no immunoreactivity, no glomerulonephritis	
				Lung	negative staining, no immunoreactivity	
				Marrow	negative staining, no immunoreactivity	
				L.Node	negative staining, no immunoreactivity	
				Spleen	negative staining, no immunoreactivity	
				Testes	negative staining, no immunoreactivity	
				Liver	negative staining, no immunoreactivity	
8	2/23/00	MRL/MpJ-fas_{LPR}	ϕ Amp1x 02/02/00	Brain	negative staining, no immunoreactivity	male
				Diaphragm	negative staining, no immunoreactivity	
				Heart	negative staining, no immunoreactivity	
				Kidney	negative staining, no immunoreactivity	
				Lung	negative staining, no immunoreactivity	
				Marrow	no marrow found on slide	
				L.Node	negative staining, no immunoreactivity	
				Spleen	negative staining, no immunoreactivity	
				Testes	negative staining, no immunoreactivity	
				Liver	negative staining, no immunoreactivity	

Study II IHC

3 Days Post Injection with Phage Amplified Twice (from tumor 2 excised from Survival Surgery Mouse #2 on 2-2-00)						
Using Rabbit α-M13 at 1:10,000 dilution (Sigma cat #B7786, lot #038H4885)						
Scale used to grade staining is - to +++						
			Phage			
Mouse	Harvested	Strain	Injection	Tissue	Comments	Sex
9	2/7/00	MRL/MpJ-fas$_{LPR}$	ϕ Amp2x 2/4/00	Brain	negative staining, no immunoreactivity	female
				Diaphragm	negative staining, no immunoreactivity	
				Heart	negative staining, no immunoreactivity	
				Kidney	**focal staining in histiocytes (?artifact)**	
				Lung	negative staining, no immunoreactivity	
				Marrow	negative staining, no immunoreactivity	
				L.Node	negative staining, no immunoreactivity	
				Spleen	negative staining, no immunoreactivity	
				Uterus	negative staining, no immunoreactivity	
				Liver	**diffuse + staining in Kupffer cells**	
10	2/7/00	MRL/MpJ-fas$_{LPR}$	ϕ Amp2x 2/4/00	Brain	negative staining, no immunoreactivity	male
				Diaphragm	negative staining, no immunoreactivity	
				Heart	negative staining, no immunoreactivity	
				Kidney	**+/++ staining in glomeruli**	
				Lung	negative staining, no immunoreactivity	
				Marrow	negative staining, no immunoreactivity	
				L.Node	negative staining, no immunoreactivity	
				Spleen	**+ staining in germinal center lymphocytes and 1+ in mantel zone**	
					and focally in endothelial cells	
				Testes	negative staining, no immunoreactivity	
				Liver	**diffuse + staining in Kupffer cells**	
11	2/7/00	FVB	ϕ Amp2x 2/4/00	Brain	negative staining, no immunoreactivity	male
				Diaphragm	negative staining, no immunoreactivity	
				Heart	**focal endothelial staining**	
				Kidney	negative staining, no immunoreactivity	
				Lung	negative staining, no immunoreactivity	
				Marrow	negative staining, no immunoreactivity	
				L.Node	**focal ++ staining within germinal center folicles**	
					and diffuse + staining in sinuses	
				Spleen	**++ staining in germinal centers,**	
					patchy ++ staining in mantel zone and sinuses	
				Testes	negative staining, no immunoreactivity	
				Liver	**diffuse + staining in Kupffer cells**	
12	2/7/00	FVB	ϕ Amp2x 2/4/00	Brain	negative staining, no immunoreactivity	male
				Diaphragm	negative staining, no immunoreactivity	
				Heart	negative staining, no immunoreactivity	
				Kidney	negative staining, no immunoreactivity	
				Lung	negative staining, no immunoreactivity	
				Marrow	negative staining, no immunoreactivity	
				L.Node	**focal moderate staining of germinal center lymphocytes**	
				Spleen	mild hemosiderin in sinusoidal histiocytes	
				Testes	negative staining, no immunoreactivity	
				Liver	**diffuse, mild to moderate Kupffer staining,**	
					(not increased in areas of inflammation)	
Positive control tissues from mouse injected with phage 10 minutes before organ harvest						
	2/7/00	MRL/MpJ-fas$_{LPR}$	2/7/00	Blood	**+staining**	
				Liver	**vascular staining**	
				Spleen	**sinusoidal staining**	

Study II IHC

3 Weeks Post Injection with Phage Amplified Twice (from tumor 2 excised from Survival Surgery Mouse #2 on 2-2-00)						
Using Rabbit α-M13 at 1:10,000 dilution (Sigma cat #B7786, lot #038H4885)						
Scale used to grade staining is - to +++						
Mouse	Harvested	Strain	Phage Injection	Tissue	Comments	Sex
13	2/24/00	MRL/MpJ-fas$_{LPR}$	ϕ Amp2x 2/4/00	Brain	negative staining, no immunoreactivity	male
				Diaphragm	negative staining, no immunoreactivity	
				Heart	negative staining, no immunoreactivity	
				Kidney	negative staining, no immunoreactivity	
				Lung	negative staining, no immunoreactivity	
				Marrow	negative staining, no immunoreactivity	
				L.Node	negative staining, no immunoreactivity	
				Spleen	negative staining, no immunoreactivity	
				Testes	negative staining, no immunoreactivity	
				Liver	negative staining, no immunoreactivity	
14	2/24/00	MRL/MpJ-fas$_{LPR}$	ϕ Amp2x 2/4/00	Brain	negative staining, no immunoreactivity	female,
				Diaphragm	negative staining, no immunoreactivity	pregnant
				Heart	negative staining, no immunoreactivity	
				Kidney	negative staining, no immunoreactivity	
				Lung	negative staining, no immunoreactivity	
				Marrow	negative staining, no immunoreactivity	
				L.Node	negative staining, no immunoreactivity	
				Spleen	negative staining, no immunoreactivity	
				Uterus	no uterus obtained	
				Liver	negative staining, no immunoreactivity	
15	2/24/00	FVB	ϕ Amp2x 2/4/00	Brain	negative staining, no immunoreactivity	female
				Diaphragm	negative staining, no immunoreactivity	
				Heart	negative staining, no immunoreactivity	
				Kidney	negative staining, no immunoreactivity	
				Lung	negative staining, no immunoreactivity	
				Marrow	negative staining, no immunoreactivity	
				L.Node	**focal staining in interfolicular lymphocytes and macrophages**	
				Spleen	negative staining, no immunoreactivity	
				Uterus	negative staining, no immunoreactivity	
				Liver	negative staining, no immunoreactivity	
16	2/24/00	FVB	ϕ Amp2x 2/4/00	Brain	negative staining, no immunoreactivity	female
				Diaphragm	negative staining, no immunoreactivity	
				Heart	negative staining, no immunoreactivity	
				Kidney	negative staining, no immunoreactivity	
				Lung	negative staining, no immunoreactivity	
				Marrow	negative staining, no immunoreactivity	
				L.Node	negative staining, no immunoreactivity	
				Spleen	negative staining, no immunoreactivity	
				Uterus	negative staining, no immunoreactivity	
				Liver	**focal weak staining in Kupffer cells**	

H&E Results: Study II[1]

	3 Days After φ Amp1x Injection				3 Weeks After φ Amp1x Injection			
Mouse	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Tissue								
Liver	NL	NL	NL	NL	NL	NL	NL	NL
Spleen	NL	NL	NL	NL	NL	NL	NL	NL
Lymph node	NL	NL	NL	NL	NL	NL	NL	NL
Gonads	NL	NL	NL	NL	NL	NL	NL	NL
Heart	NL	NL	NL	NL	NL	NL	NL	NL
Lung	NL	NL	NL	NL	NL	NL	NL	NL
Bone marrow	NL	NL	NL	NL	NL	NL	NL	NL
Kidney	NL	NL	NL	NL	NL	NL	NL	NL
Brain	NL	NL	NL	NL	NL	NL	NL	NL
Diaphragm	NL	NL	NL	NL	NL	NL	NL	NL

	3 Days After φ Amp1x Injection				3 Weeks After φ Amp1x Injection			
Mouse	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**
Tissue								
Liver	NL	NL	∅[2,6]	∅[3,6]	NL	NL	∅[4,6]	∅[5,6]
Spleen	NL	NL	NL	NL	NL	NL	NL	NL
Lymph node	NL	NL	NL	NL	NL	NL	NL	NL
Gonads	NL	NL	NL	NL	NL	NL	NL	NL
Heart	NL	NL	NL	NL	NL	NL	NL	NL
Lung	NL	NL	NL	NL	NL	NL	NL	NL
Bone marrow	NL	NL	NL	NL	NL	NL	NL	NL
Kidney	NL	NL	NL	NL	NL	NL	NL	NL
Brain	NL	NL	NL	NL	NL	NL	NL	NL
Diaphragm	NL	NL	NL	NL	NL	NL	NL	NL

[1]NL denotes normal histology for the strain

[2]Focal acute lobular hepatitis. No evidence of fibrosis. Dead hepatocytes/tissue injury. Focal process diffusely distributed in small foci. Each nidus consists of apoptotic and degenerating hepatocytes primarily associated with neutrophils in the larger foci.

[3]Chronic and focally acute lobular hepatitis, less severe but similar pattern to mouse 11.

[4]Mild chronic lobular hepatitis, focal mild cholestasis, no necrotic hepatocytes.

[5]Lymphoid aggregates.

[6]Subsequently stained with Steiner silver stain to rule out Helicobacter or Clostridium infection. No bacteria were identified.

Study II H&E

3 Days After Injection with Phage Amplified Once (from tumor 1 excised from Survival Surgery Mouse #2 on 1-31-00)							
Mouse	Harvested	Strain	Phage Injection	Tissue	Pathology	Comments	Sex
1	2/5/00	FVB	ϕAmp1x 2/2/00	Brain	not available	animal died in restraint prior to	
				Diaphragm	not available	tail vein injection	
				Heart	not available		
				Kidney	not available		
				Lung	not available		
				Marrow	not available		
				L.Node	not available		
				Spleen	not available		
				Testes	not available		
				Liver	not available		
2	2/5/00	FVB	ϕAmp1x 2/2/00	Brain	normal		female
				Diaphragm	normal		
				Heart	normal		
				Kidney	normal		
				Lung	normal		
				Marrow	normal		
				L.Node	normal		
				Spleen	normal	mild hemosiderin in histocytes, diffusely distributed	
				Uterus	normal		
				Liver	normal		
3	2/5/00	MRL/MpJ-fas$_{LPR}$	ϕAmp1x 2/2/00	Brain	normal		
				Diaphragm	normal	focal lymphoid aggregates	male
				Heart	normal		
				Kidney	normal		
				Lung	normal		
				Marrow	not available	no marrow found on slide	
				L.Node	normal		
				Spleen	normal		
				Testes	normal		
				Liver	normal		
4	2/5/00	MRL/MpJ-fas$_{LPR}$	ϕAmp1x 2/2/00	Brain	normal		female
				Diaphragm	normal	focal lymphoid aggregates	
				Heart	normal		
				Kidney	normal	chronic interstitial nephritis, glomerular sclerosis	
				Lung	normal		
				Marrow	normal		
				L.Node	normal		
				Spleen	normal		
				Uterus	normal		
				Liver	normal		
Positive Control Tissues from mouse injected with phage 10 minutes before tissue harvest:							
	2/5/00	MRL/MpJ-fas$_{LPR}$	ϕ 2/5/00	Blood	normal		
				Lung	normal		
				Spleen	normal		

Study II H&E

3 Weeks After Injection with Phage Amplified Once (from tumor 1 excised from Survival Surgery Mouse #2 1-31-00)							
Mouse	Harvested	Strain	Phage Injection	Tissue	Pathology	Comments	Sex
5	2/23/00	FVB	ϕAmp1x 2/2/00	Brain	normal		female
				Diaphragm	normal		
				Heart	normal		
				Kidney	normal		
				Lung	normal		
				Marrow	normal		
				L.Node	normal		
				Spleen	normal		
				Uterus	normal		
				Liver	normal		
6	2/23/00	FVB	ϕAmp1x 2/2/00	Brain	normal		female
				Diaphragm	normal		
				Heart	normal		
				Kidney	normal	minimal focal chronic tubular inflammation	
				Lung	normal		
				Marrow	normal		
				L.Node	normal		
				Spleen	normal		
				Testes	normal		
				Liver	normal		
7	2/23/00	MRL/MpJ-fas_{LPR}	ϕAmp1x 2/2/00	Brain	normal		male
				Diaphragm	normal		
				Heart	normal		
				Kidney	normal	no glomerulonephritis	
				Lung	normal		
				Marrow	normal		
				L.Node	normal		
				Spleen	normal		
				Testes	normal		
				Liver	normal		
8	2/23/00	MRL/MpJ-fas_{LPR}	ϕAmp1x 2/2/00	Brain	normal		male
				Diaphragm	normal		
				Heart	normal		
				Kidney	normal		
				Lung	normal		
				Marrow	not available	no marrow found on slide	
				L.Node	normal		
				Spleen	normal		
				Testes	normal		
				Liver	normal		
Positive Control Tissues from mouse injected with phage 10 minutes before tissue harvest:							
	2/23/00	FVB	ϕ 2/23/00	Blood			
				Liver			
				Spleen			
				Heart			

Study II H&E

3 Days After Injection with Phage Amplified Twice (from tumor 2 excised from Survival Surgery Mouse #2 on 2-2-00)							
Mouse	Harvested	Strain	Phage Injection	Tissue	Pathology	Comments	Sex
9	2/7/00	MRL/MpJ-fas$_{LPR}$	ϕAmp2x 2/4/00	Brain	normal		female
				Diaphragm	normal		
				Heart	normal		
				Kidney	normal	interstitial nephritis + focal glomerular sclerosi	
				Lung	normal	lympho proliferative infiltrates	
				Marrow	normal		
				L.Node	normal		
				Spleen	normal		
				Uterus	normal		
				Liver	normal		
10	2/7/00	MRL/MpJ-fas$_{LPR}$	ϕAmp2x 2/4/00	Brain	normal		male
				Diaphragm	normal		
				Heart	normal		
				Kidney	normal	no glomerulonephritis	
				Lung	normal		
				Marrow	normal		
				L.Node	normal		
				Spleen	normal		
				Testes	normal		
				Liver	normal		
11	2/7/00	FVB	ϕAmp2x 2/4/00	Brain	normal		male
				Diaphragm	normal		
				Heart	normal		
				Kidney	normal		
				Lung	normal		
				Marrow	normal		
				L.Node	normal		
				Spleen	normal		
				Testes	normal		
				Liver		See note (1) below.	
Note (1): Focal acute lobular hepatitis. No evidence of fibrosis, dead hepatocytes/tissue injury, focal process diffusely distributed in sm							
within the lobules and occasionally involving portal triads.							
Each nidus consists of apoptotic and degenerating hepatocytes primarily associated with neutrophils in the larger foci.							
Could be an immune mediated process.							
12	2/7/00	FVB	ϕAmp2x 2/4/00	Brain	normal		male
				Diaphragm	normal		
				Heart	normal		
				Kidney	normal		
				Lung	normal		
				Marrow	normal		
				L.Node	normal		
				Spleen	normal		
				Testes	normal		
				Liver		See note (2) below.	
Note (2): Chronic and focally acute lobular hepatitis, less severe but similar pattern to mouse 11.							
Positive Control Tissues from mouse injected with phage 10 minutes before tissue harvest:							
	2/7/00	MRL/MpJ-fas$_{LPR}$	ϕ 2/7/00	Blood	normal		
				Liver	normal		
				Spleen	normal		

Study II H&E

3 Weeks Post Injection with Phage Amplified Twice (from tumor 2 excised from Survival Surgery Mouse #2 on 2-2-00)							
Mouse	Harvested	Strain	Phage Injection	Tissue	Pathology	Comments	Sex
13	2/24/00	MRL/MpJ-fas$_{LPR}$	ϕAmp2x 2/4/00	Brain	normal		male
				Diaphragm	normal		
				Heart	normal		
				Kidney	normal		
				Lung	normal		
				Marrow	normal		
				L.Node	normal		
				Spleen	normal		
				Testes	normal		
				Liver	normal		
14	2/24/00	MRL/MpJ-fas$_{LPR}$	ϕAmp2x 2/4/00	Brain	normal		female, pre
				Diaphragm	normal		
				Heart	normal		
				Kidney	normal	patchy interstitial nephritis	
				Lung	normal		
				Marrow	normal		
				L.Node	normal		
				Spleen	normal	more prominent extra medullary hematopoiesis,	
						stimulated white pulp (folicular hyperplasia)	
				Uterus	not available	technicians did not know animal was pregnant until after t	
				Liver	normal		
15	2/24/00	FVB	ϕAmp2x 2/4/00	Brain	normal		female
				Diaphragm	normal		
				Heart	normal		
				Kidney	normal		
				Lung	normal		
				Marrow	normal		
				L.Node	normal		
				Spleen	normal	moderate hemosiderin sinusodal histocytes	
				Uterus	normal		
				Liver		**See note (3) below.**	
Note (3): Mild chronic lobular hepatitis, focal mild cholestasis, no necrotic hepatocytes.							
16	2/24/00	FVB	ϕAmp2x 2/4/00	Brain	normal		female
				Diaphragm	normal		
				Heart	normal		
				Kidney	normal		
				Lung	normal		
				Marrow	normal		
				L.Node	normal		
				Spleen	normal	moderate hemosiderin sinusodal histocytes	
				Uterus	normal	neutrophils in myometrium and endometrial stroma	
						? estrus cycle ?	
				Liver	normal		

Study III

Toxicity Study in Mice Following Three Injections of Phage: Naïve, Amp1x, Amp2x

Diagram of Study Design: Study III

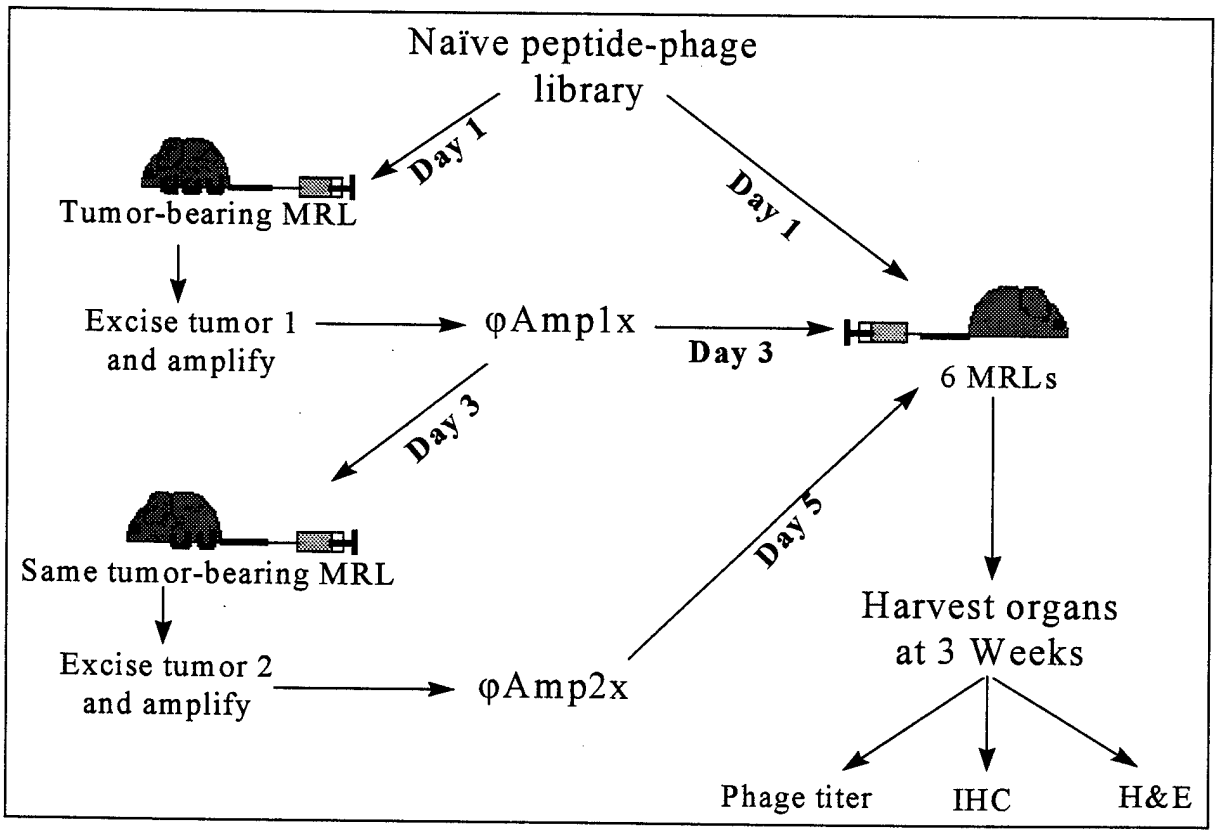


Table of Study Design: Study III

Mouse	Strain	Three φ Injections at 48 hr Intervals: naïve, φ Amp1x, φ Amp2x	Organ Harvest at 3 Weeks
1	MRL	+	X
2	MRL	+	X
3	MRL	+	X
4	MRL	+	X
5	MRL	+	X
6	MRL	+	X

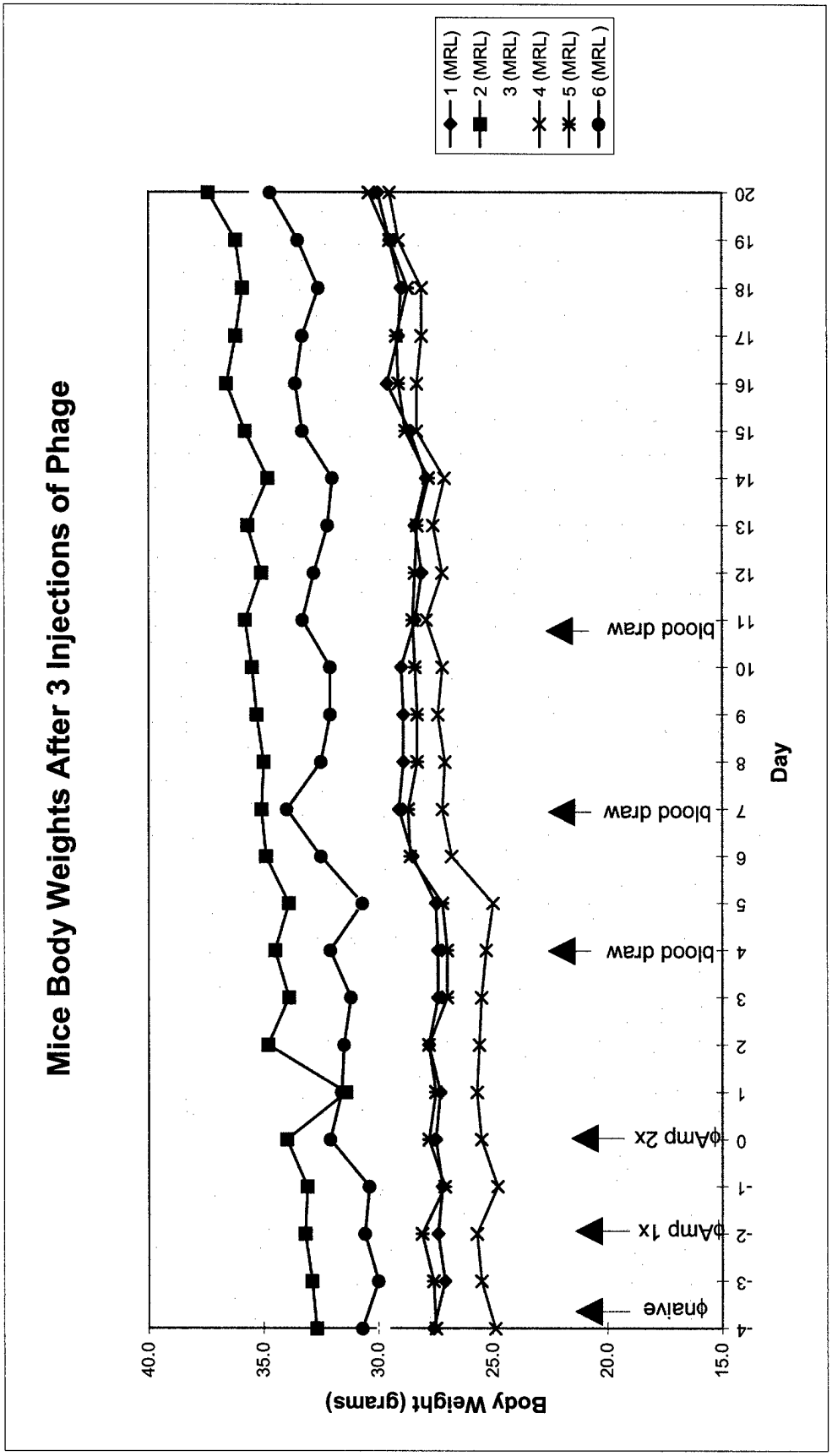
Methods: Study III

- On 4/3/00, 6 mice (MRL/MpJ-fasLPR) were injected via the tail vein with 1.5×10^{10} naïve peptide-phage in a volume of 250 µl.
- On 4/5/00, the same mice were injected again with 1.4×10^{10} φAmp1x, in a volume of 250 µl. φAmp1x were peptide-phage amplified from tumor 1 which was excised from Survival Surgery Mouse #3 on 4/3/00. See Study IV for details on survival surgery mice.
- On 4/7/00, the same mice were injected again with 1.4×10^{10} φAmp2x, in a volume of 200 µl. φAmp2x were peptide-phage amplified from tumor 2 which was excised from Survival Surgery Mouse #3 on 4/5/00.
- Mice were monitored daily after peptide-phage injection until organ harvesting. During the monitoring period, mice were weighed and observed for signs of toxicity.
- Blood was drawn 4 days following injection, and then twice a week thereafter, until blood was shown to be clear of infective phage by titering.
- The mice were euthanized at 3 weeks. Immediately following euthanasia, samples from ten organs (brain, diaphragm, heart, kidney, lung, bone marrow, lymph node, spleen , gonads, and liver) were harvested, placed in buffered formalin and sent to FAHC Histology for processing, H&E staining, and IHC staining. All slides were subsequently read by the project pathologist.
- The same tissues were also collected (all but marrow, due to small sample size) for phage titering, future PCR, and confocal analysis.

Summary of Results: Study III

- **Survival:** All mice survived to the end point.
- **Gross appearance:** Activity, behavior, and appearance were observed to be normal in all mice for the duration of the study.
- **Weights:** Mice weights appeared to either remain the same or increase (consistent with normal growth/control mouse) over the course of the study.
- **Phage Titers:** Blood was free of infective phage 11 days after the third and final injection of peptide-phage. No infective phage were detected in any of the tissues collected three weeks after the third injection of phage.
- **IHC:** All tissues were negative for phage three weeks following phage injection.
- **H&E:** All collected tissues were found to be of normal histology for the strain.

Study III


Mice Body Weights After 3 Injections of Phage
Body Weight (grams)
40.0
35.0
30.0
25.0
20.0
15.0
Day
-4
-3
-2
-1
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
ϕnaive
ϕAmp 1x
ϕAmp 2x
blood draw
blood draw
blood draw
1 (MRL)
2 (MRL)
3 (MRL)
4 (MRL)
5 (MRL)
6 (MRL)

Study III

Body Weights for Mice								
			Date of phage injection:					
			04/03/00	04/03/00	04/03/00	04/03/00	04/03/00	04/03/00
			04/05/00	04/05/00	04/05/00	04/05/00	04/05/00	04/05/00
			04/07/00	04/07/00	04/07/00	04/07/00	04/07/00	04/07/00
			Mouse					
Date	**Day**		1 (MRL)	2 (MRL)	3 (MRL)	4 (MRL)	5 (MRL)	6 (MRL)
04/03/00	-4		27.6	32.7	29.8	24.9	27.5	30.7
04/04/00	-3		27.1	32.9	29.4	25.5	27.6	30.0
04/05/00	-2		27.4	33.2	30.6	25.7	28.1	30.6
04/06/00	-1		27.2	33.1	30.2	24.8	27.1	30.4
04/07/00	0		27.5	34.0	30.9	25.5	27.8	32.1
04/08/00	1		27.3	31.4	30.5	25.7	27.5	31.6
04/09/00	2		27.8	34.8	30.7	25.6	27.8	31.5
04/10/00	3		27.4	33.9	30.7	25.5	27.0	31.2
04/11/00	4		27.4	34.5	31.6	25.3	27.0	32.1
04/12/00	5		27.5	33.9	31.1	25.0	27.2	30.7
04/13/00	6		28.5	34.9	32.7	26.8	28.6	32.5
04/14/00	7		29.1	35.1	32.6	27.2	28.7	34.0
04/15/00	8		28.9	35.0	32.3	27.1	28.3	32.5
04/16/00	9		28.9	35.3	32.3	27.4	28.3	32.1
04/17/00	10		29.0	35.5	32.8	27.2	28.4	32.1
04/18/00	11		28.4	35.8	34.0	27.9	28.5	33.3
04/19/00	12		28.1	35.1	33.0	27.2	28.4	32.8
04/20/00	13		28.4	35.7	32.7	27.6	28.3	32.2
04/21/00	14		27.9	34.8	32.2	27.1	27.8	32.0
04/22/00	15		28.6	35.8	32.6	28.3	28.8	33.3
04/23/00	16		29.6	36.6	33.7	28.3	29.1	33.6
04/24/00	17		29.1	36.2	34.3	28.1	29.2	33.3
04/25/00	18		29.0	35.9	33.7	28.1	28.7	32.6
04/26/00	19		29.5	36.2	34.5	29.1	29.5	33.5
04/27/00	20		30.0	37.4	35.3	29.5	30.4	34.7

Phage Titer Results for Blood Clearance: Study III

Days After Final Phage Injection	Mouse	Volume of Blood (µl)	Transducing Units (TU)	TU/ml_{blood}
4	1	3	6	2
	2	5	6	1.2
	3	3	0	0
	4	3	0	0
	5	1	1	0.3
	6	3	0	0
7	1	3	0	0
	2	6	1	0.2
	3	3	0	0
	4	6	1	0.2
	5	2	0	0
	6	5	0	0
11	1	20	0	0
	2	17	0	0
	3	20	0	0
	4	15	0	0
	5	20	0	0
	6	20	0	0

Phage Titer Results: Study III[1]

3 Weeks After Three Injections

Mouse	1	2	3	4	5	6
Tissue						
Liver	-	-	-	-	-	-
Spleen	-	-	-	-	-	-
Lymph node	-	-	-	-	-	-
Gonads	-	-	-	-	-	-
Heart	-	-	-	-	-	-
Lung	-	-	-	-	-	-
Bone marrow	-	-	-	-	-	-
Kidney	-	-	-	-	-	-
Brain	-	-	-	-	-	-
Diaphragm	-	-	-	-	-	-

[1]+/- denotes the presence/absence of infective phage in the tissue.

IHC Results: Study III[1]

3 Weeks After Three Injections

Mouse 1	2	3	4	5	6
Tissue					
Liver -	-	-	-	-	-
Spleen -	-	-	-	-	-
Lymph node -	-	NA[2]	-	-	-
Gonads -	-	-	-	-	-
Heart -	-	-	-	-	-
Lung -	-	-	-	-	-
Bone marrow -	-	-	-	-	-
Kidney -	-	-	-	-	-
Brain -	-	-	-	-	-
Diaphragm -	-	-	-	-	-

[1]Note a scale of - to +++ used to grade staining.
[2]Not available, no nodal tissue on slide, only brown fat which was negative for immunoreactivity.

Study III IHC

3 Weeks After 3 Injections of Phage: naïve library injected 4/3/00, phage amplified once (from tumor 1 excised 4/3/00						
from Survival Surgery Mouse 3) injected 4/5/00, and phage amplified twice (from tumor 2 excised 4/5/00 from Survival Surgery Mouse 3) injected 4/7/00.						
Primary antibody=Rabbit α-M13 at 1:10,000 dilution (Sigma cat #B7786, lot#038H4885)						
Scale used to grade staining is - to +++						
			Phage			
Mouse	**Harvested**	**Strain**	**Injection**	**Tissue**	**Comments**	**Sex**
1	04-27-00	MRL/MpJ-fas$_{LPR}$	ϕ naive 4/3/00	Brain	negative staining, no immunoreactivity	female
			ϕAmp1x 4/5/00	Diaphragm	negative staining, no immunoreactivity	
			ϕAmp2x 4/7/00	Heart	negative staining, no immunoreactivity	
				Kidney	negative staining, no immunoreactivity	
				Lung	negative staining, no immunoreactivity	
				Marrow	negative staining, no immunoreactivity	
				L.Node	negative staining, no immunoreactivity	
				Spleen	negative staining, no immunoreactivity	
				Uterus	negative staining, no immunoreactivity	
				Liver	negative staining, no immunoreactivity	
2	04-27-00	MRL/MpJ-fas$_{LPR}$	ϕ naive 4/3/00	Brain	negative staining, no immunoreactivity	female
			ϕAmp1x 4/5/00	Diaphragm	negative staining, no immunoreactivity	
			ϕAmp2x 4/7/00	Heart	negative staining, no immunoreactivity	
				Kidney	negative staining, no immunoreactivity	
				Lung	negative staining, no immunoreactivity	
				Marrow	negative staining, no immunoreactivity	
				L.Node	negative staining, no immunoreactivity	
				Spleen	negative staining, no immunoreactivity	
				Uterus	negative staining, no immunoreactivity	
				Liver	negative staining, no immunoreactivity	
3	04-27-00	MRL/MpJ-fas$_{LPR}$	ϕ naive 4/3/00	Brain	negative staining, no immunoreactivity	female
			ϕAmp1x 4/5/00	Diaphragm	negative staining, no immunoreactivity	
			ϕAmp2x 4/7/00	Heart	negative staining, no immunoreactivity	
				Kidney	negative staining, no immunoreactivity	
				Lung	negative staining, no immunoreactivity	
				Marrow	negative staining, no immunoreactivity	
				L.Node	no nodal tissue, only brown fat, negative staining, no immunoreactivity	
				Spleen	negative staining, no immunoreactivity	
				Uterus	negative staining, no immunoreactivity	
				Liver	negative staining, no immunoreactivity	

Study III IHC

3 Weeks Following 3 Injections of Phage: naïve library injected 4/3/00, phage amplified once (from tumor 1 excised 4/3/00							
from Survival Surgery Mouse 3) injected 4/5/00, and phage amplified twice (from tumor 2 excised 4/5/00 from Survival Surgery Mouse 3) injected 4/7/00.							
Primary antibody=Rabbit α-M13 at 1:10,000 dilution (Sigma cat #B7786, lot#038H4885)							
Scale used to grade staining is - to +++							
			Phage				
Mouse	**Harvested**	**Strain**	**Injection**	**Tissue**	**Comments**		**Sex**
4	04-27-00	MRL/MpJ-fas$_{LPR}$	ϕ naive 4/3/00	Brain	negative staining, no immunoreactivity		female
			ϕAmp1x 4/5/00	Diaphragm	negative staining, no immunoreactivity		
			ϕAmp2x 4/7/00	Heart	negative staining, no immunoreactivity		
				Kidney	negative staining, no immunoreactivity		
				Lung	negative staining, no immunoreactivity		
				Marrow	negative staining, no immunoreactivity		
				L.Node	negative staining, no immunoreactivity		
				Spleen	negative staining, no immunoreactivity		
				Uterus	negative staining, no immunoreactivity		
				Liver	negative staining, no immunoreactivity		
5	04-27-00	MRL/MpJ-fas$_{LPR}$	ϕ naive 4/3/00	Brain	negative staining, no immunoreactivity		female
			ϕAmp1x 4/5/00	Diaphragm	negative staining, no immunoreactivity		
			ϕAmp2x 4/7/00	Heart	negative staining, no immunoreactivity		
				Kidney	negative staining, no immunoreactivity		
				Lung	negative staining, no immunoreactivity		
				Marrow	negative staining, no immunoreactivity		
				L.Node	negative staining, no immunoreactivity		
				Spleen	negative staining, no immunoreactivity		
				Uterus	negative staining, no immunoreactivity		
				Liver	negative staining, no immunoreactivity		
6	04-27-00	MRL/MpJ-fas$_{LPR}$	ϕ naive 4/3/00	Brain	negative staining, no immunoreactivity		female
			ϕAmp1x 4/5/00	Diaphragm	negative staining, no immunoreactivity		
			ϕAmp2x 4/7/00	Heart	negative staining, no immunoreactivity		
				Kidney	negative staining, no immunoreactivity		
				Lung	negative staining, no immunoreactivity		
				Marrow	negative staining, no immunoreactivity		
				L.Node	negative staining, no immunoreactivity		
				Spleen	negative staining, no immunoreactivity		
				Uterus	negative staining, no immunoreactivity		
				Liver	negative staining, no immunoreactivity		

H&E Results: Study III[1]

3 Weeks After Three Injections

Mouse	1	2	3	4	5	6
Tissue						
Liver	NL	NL	NL	NL	NL	NL
Spleen	NL	NL	NL	NL	NL	NL
Lymph node	NL	NL	NA[2]	NL	NL	NL
Gonads	NL	NL	NL	NL	NL	NL
Heart	NL	NL	NL	NL	NL	NL
Lung	NL	NL	NL	NL	NL	NL
Bone marrow	NL	NL	NL	NL	NL	NL
Kidney	NL	NL	NL	NL	NL	NL
Brain	NL	NL	NL	NL	NL	NL
Diaphragm	NL	NL	NL	NL	NL	NL

[1]NL denotes normal histology for the strain.
[2]Not available, no nodal tissue was found, only brown fat.

Study III H&E

3 Weeks After 3 Injections of Phage: naïve library injected 4/3/00, phage amplified once (from tumor 1 excised 4/3/00							
from Survival Surgery Mouse #3) injected 4/5/00, and phage amplified twice (from tumor 2 excised 4/5/00 from Survival Surgery Mouse #3) injected 4/7/00.							
			Phage				
Mouse	**Harvested**	**Strain**	**Injection**	**Tissue**	**Pathology**	**Comments**	**Sex**
1	04-27-00	MRL/MpJ-fas$_{LPR}$	ϕ naive 4/3/00	Brain	normal		female
			ϕAmp1x 4/5/00	Diaphragm	normal		
			ϕAmp2x 4/7/00	Heart	normal		
				Kidney	normal		
				Lung	normal		
				Marrow	normal		
				L.Node	normal	slide has node, pancreas, and brown fat	
				Spleen	normal	extramedually hematopoiesis	
				Uterus	normal		
				Liver	normal		
2	04-27-00	MRL/MpJ-fas$_{LPR}$	ϕ naive 4/3/00	Brain	normal		female
			ϕAmp1x 4/5/00	Diaphragm	normal		
			ϕAmp2x 4/7/00	Heart	normal		
				Kidney	normal		
				Lung	normal		
				Marrow	normal		
				L.Node	normal		
				Spleen	normal		
				Uterus	normal		
				Liver	normal		
3	04-27-00	MRL/MpJ-fas$_{LPR}$	ϕ naive 4/3/00	Brain	normal		female
			ϕAmp1x 4/5/00	Diaphragm	normal		
			ϕAmp2x 4/7/00	Heart	normal		
				Kidney	normal	minimal interstitial lymphoid aggregates	
				Lung	normal		
				Marrow	normal		
				L.Node	not available	no nodal tissue obtained, only brown fat	
				Spleen	normal		
				Uterus	normal		
				Liver	normal		

Study III H&E

3 Weeks After 3 Injections of Phage: naïve library injected 4/3/00, phage amplified once (from tumor 1 excised 4/3/00							
from Survival Surgery Mouse #3) injected 4/5/00, and phage amplified twice (from tumor 2 excised 4/5/00 from Survival Surgery Mouse #3) injected 4/7/00.							
			Phage				
Mouse	**Harvested**	**Strain**	**Injection**	**Tissue**	**Normal Path?**	**Comments**	**Sex**
4	04-27-00	MRL/MpJ-fas$_{LPR}$	ϕ naive 4/3/00	Brain	normal		female
			ϕAmp1x 4/5/00	Diaphragm	normal		
			ϕAmp2x 4/7/00	Heart	normal		
				Kidney	normal	mild interstitial nephritis	
				Lung	normal		
				Marrow	normal		
				L.Node	normal		
				Spleen	normal	extramedullary hematopoiesis	
				Uterus	normal		
				Liver	normal		
5	04-27-00	MRL/MpJ-fas$_{LPR}$	ϕ naive 4/3/00	Brain	normal		female
			ϕAmp1x 4/5/00	Diaphragm	normal		
			ϕAmp2x 4/7/00	Heart	normal		
				Kidney	normal	mild interstitial lymphoid aggregates	
				Lung	normal		
				Marrow	normal		
				L.Node	normal		
				Spleen	normal		
				Uterus	normal		
				Liver	normal		
6	04-27-00	MRL/MpJ-fas$_{LPR}$	ϕ naive 4/3/00	Brain	normal		female
			ϕAmp1x 4/5/00	Diaphragm	normal		
			ϕAmp2x 4/7/00	Heart	normal		
				Kidney	normal	mild chronic interstitial nephritis	
				Lung	normal		
				Marrow	normal		
				L.Node	normal		
				Spleen	normal		
				Uterus	normal		
				Liver	normal		

Group IV
Toxicity Study in Mice Following In Vivo Screening

Diagram of Design: Study IV

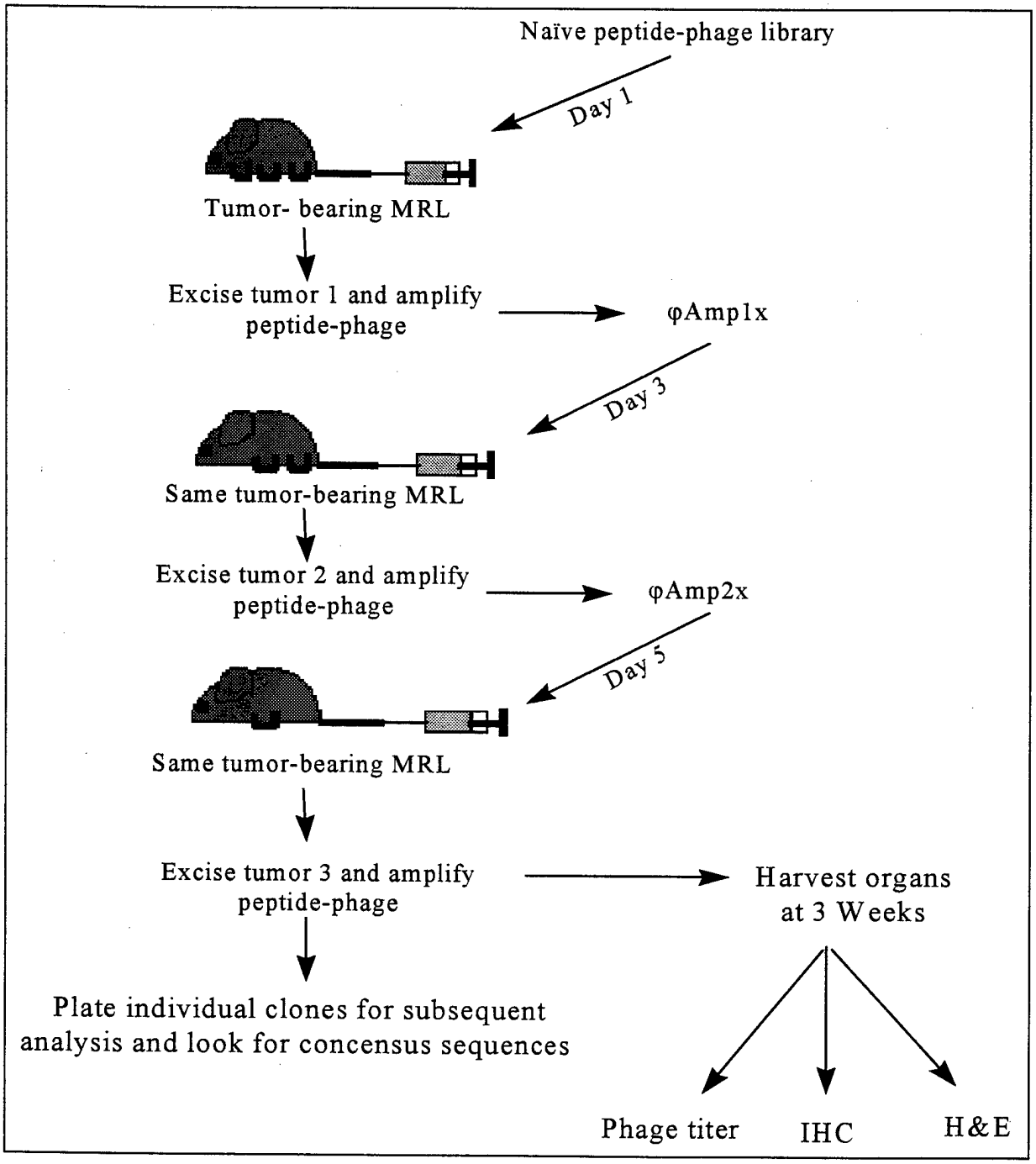


Table of Study Design: Study IV

Mouse	Strain	Three φ injections: naïve, φ Amp1x, φ Amp2x	Organ Harvest at 3 Weeks
1	MRL	+	X
2	MRL	+	X
3	MRL	+	X
4	MRL (control)	-	X

Methods: Study IV

- On the first day of surgery, a MRL mouse bearing at least 3 palpable tumors was anesthetized with halothane and injected IV with a peptide-phage suspension via the tail vein. The phage were allowed to circulate for 10 minutes. Under aseptic conditions, tumor 1 was excised. The incision was sutured and the mouse allowed to recover. The tumor was weighed, minced, ground, and the phage recovered. Peptide-phage were then amplifed and labeled as "φ Amp1x." (Refer to Appendix for Surgical Protocol.)
- On the second day of surgery, (1-2 days following the first surgery), the mouse was again anesthetized with halothane, injected with φ Amp1x (amplified from tumor 1), and allowed to circulate for 10 minutes. Tumor 2 was excised as before, the incision sutured, and the mouse allowed to recover. Tumor 2 was ground and amplified as before and labeled as "φ Amp2x."
- On the third and final surgery day, the mouse was anesthetized as before and injected with φ Amp2x (amplified from tumor 2) and allowed to circulate 10 minutes. A third tumor was excised, the incision sutured and the animal allowed to recover. The phage were eluted from the tumor, incubated with K91 Kan *E. coli*, and plated for future DNA sequencing analysis.
- Mice were monitored daily after peptide-phage injection until organ harvesting. During the monitoring period the mice were weighed and observed for signs of toxicity.
- Mice were euthanized three weeks following the third phage injection/surgery (in vivo screening). Immediately following euthanasia, samples from ten organs (brain, diaphragm, heart, kidney, lung, bone marrow, lymph node, spleen, gonads, and liver) were harvested, placed in buffered formalin and sent to FAHC Histology for processing, H&E staining, and IHC staining. All slides were subsquently read by the project pathologist. Note that mouse #1 was injected with amplified peptide-phage a fourth time, on the day of organ harvest (after removal of a 4th tumor, and immediately prior to all other tissue collection) to provide phage-positive tissues for IHC.

Number of Peptide-Phage (TU[1]) Injected: Study IV

Mouse	Naïve	φ Amp1x	φ Amp2x	φ Amp3x
1	NA[2] (9/21/99)	NA[2] (9/22/99)	NA[2] (9/24/99)	NA[2,3] (10/15/99)
2	2.6×10^9 (1/31/00)	6.4×10^9 (2/2/00)	9.3×10^{11} (2/4/00)	
3	3.8×10^9 (4/3/00)	3.6×10^9 (4/5/00)	NA[4]	

[1]Note that only about 1 in 20 particles infect E. coli; ie can be assayed as a transducing unit (TU)[9].
[2]Not available.
[3]Peptide-phage amplified from tumor 3 (excised 9/24/99 from mouse #1) was injected on the day of organ harvest to provide phage-positive IHC tissues.
[4]Mouse died following excision of tumor on the second day of surgery while under general anesthesia.

Summary of Results: Study IV

- **Survival:** Two out of three mice (#1and #2) survived to the 3-week endpoint. The third mouse (#3), died while under general anesthesia following the removal of tumor during the second surgery. It is most likely that this animal died from excessive halothane anesthesia, due to the difficulty of monitoring the breathing and heartbeat of such a small animal, while maintaining sterile surgical technique (draping). Furthermore, as discussed previously (see Integrated Summary), the general health of the MRL tumor-bearing mice is less than optimum and may very well have played a role in Mouse #3's death during surgery. (Refer to the Appendix for Survival Surgery Protocol.)

- **Gross appearance:** The mice were somewhat less active the first day or two following each surgery. As discussed above, Surgical Mouse #3 died during the second surgery, therefore there are limited observations for this animal. Additionally, two of the surgical mice (#1and #2) pulled and chewed their sutures; Mouse #2 removed its sutures to the point of opening an incision. The incision was cleaned, and antibiotic ointment applied daily until the wound healed. The surgical mice, which were subjected to a complete phage screening, similar to the clinical protocol except with more extensive surgery and anesthesia (#1 and #2), progressed well to the end of the study. With the exception of a few days of recovery time after each surgery, the animals appeared normal for the duration of the study.

- **Weights:** The weight of Mouse #1 was measured on days 6-24 after in vivo screening and indicated a fairly stable maintenance of body weight during this time.

Mouse #2 lost 2.4 grams of body weight (5.0%) on the day following the initial surgery. After this initial loss, the weight stabilized until day 8. Mouse #2 lost 10.5% of its body weight over the next two days, but put on weight steadily through the remainder of the study, with a final weight within 5% of the starting weight.

Mouse #3 lost 3.4 grams of body weight (7.5%) in the two days following the initial surgery. No further weight measurements were made as the animal died during the second surgery. (Please refer to graph and table for details.)

- **Phage Titers:** Most tissue titers for infective phage were positive at three weeks for Mouse #2. Mouse #1 was injected with phage just before organ harvest to provide positive IHC controls, and Mouse #3 did not survive surgery 2. Therefore, titers were not performed on Mouse #1 or Mouse #3 tissues.

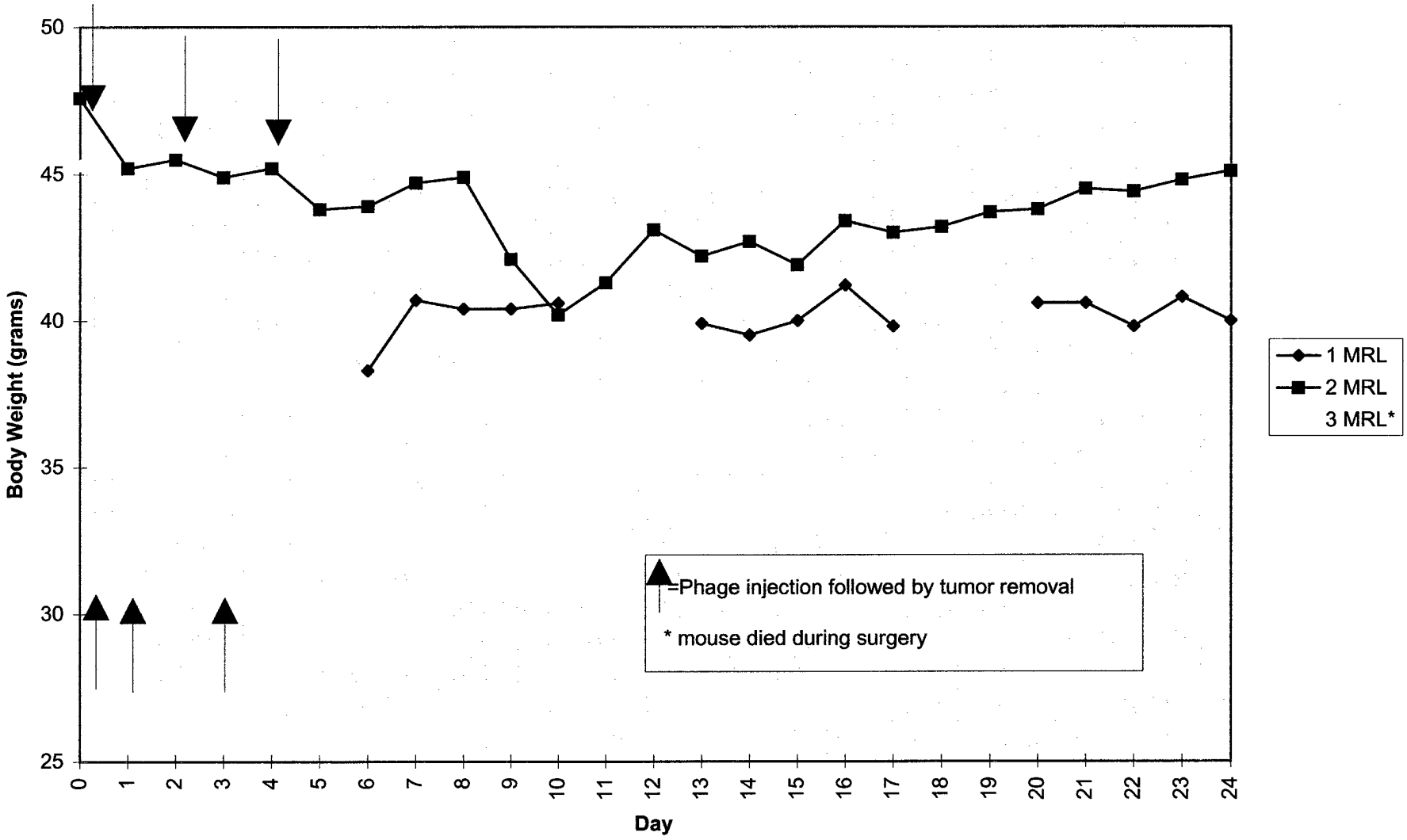
- **IHC:** Based on our titering results, we had expected to see positive IHC results. However, at the three-week endpoint, all tissues were negative for immunoreactivity in Mouse #2, indicating the absence of phage particles. This is not altogether unexpected as titering is a very sensitive technique for detection of phage, and can detect as little as 1 phage TU. In addition, far less tissue is used in the IHC technique than in titering. Far more phage particles need to be present for a positive IHC signal.

Mouse #1 was injected with phage immediately following removal of one tumor and prior to all other tissues being harvested to provide us with positive IHC controls. As expected, the

tumor removed prior to phage injection was IHC negative for phage while all other tissues were IHC positive.

- **H&E:** All tissues were determined to have normal histology for the strain.

Study IV


Body Weights of Survival Surgical Mice
Body Weight (grams)
50
45
40
35
30
25
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
Day
1 MRL
2 MRL
3 MRL*
=Phage injection followed by tumor removal
* mouse died during surgery

Study IV

Body Weights (grams) of Surgical Survival Mice

* Injection of naïve library phage, followed by surgery to remove tumor 1

** Injection with ϕAmp 1x from tumor #1, followed by surgery to remove tumor 2

***Injection with ϕAmp 2x from tumor #2 followed by surgery to remove tumor 3

	Mouse 1			Mouse 2			Mouse 3
Day			Day			Day	
*0			*0	47.6		*0	45.3
**1			1	45.2		1	42.2
2			**2	45.5		**2	41.9
3			3	44.9		3	
***4			***4	45.2		4	
5			5	43.8		5	
6	38.3		6	43.9		6	
7	40.7		7	44.7		7	
8	40.4		8	44.9		8	
9	40.4		9	42.1		9	
10	40.6		10	40.2		10	
11			11	41.3		11	
12			12	43.1		12	
13	39.9		13	42.2		13	
14	39.5		14	42.7		14	
15	40.0		15	41.9		15	
16	41.2		16	43.4		16	
17	39.8		17	43.0		17	
18			18	43.2		18	
19			19	43.7		19	
20	40.6		20	43.8		20	
21	40.6		21	44.5		21	
22	39.8		22	44.4		22	
23	40.8		23	44.8		23	
24	40.0		24	45.1		24	

Note: Mouse 3 died while under anesthesia during the second surgery.

Phage Titer Results: Study IV

3 Weeks After 3 Injections[1]
(In Vivo Screening)

Mouse	1	2	3
Tissue			
Liver	NA[2]	+	NA[3]
Spleen	NA	+	NA
Lymph node	NA	+	NA
Gonads	NA	+	NA
Heart	NA	+	NA
Lung	NA	+	NA
Kidney	NA	+	NA
Brain	NA	+	NA
Diaphragm	NA	+	NA
Tumor	NA	-	NA
Blood	NA	+	NA

[1]+/- denotes the presence/absence of infective phage in the tissue.
[2]Not available, mouse #1 was injected with phage on the day of harvest. Since phage were known to be present, titering was not performed.
[3]Not available, mouse died while still anesthetized following tumor 2 excision. No tissues were available for phage titering.

IHC Results: Study IV

3 Weeks After 3 Injections[1]
(In Vivo Screening)

Mouse	1[2]	2	3	Control MRL[4]
Tissue				
Tumor 1	-[5]	-	NA[3]	-
Liver	++	-	NA	-
Spleen	+	-	NA	-
Lymph node	NA	-	NA	-
Uterus	++	-	NA	-
Heart	+	-	NA	-
Lung	++	-	NA	-
Kidney	+	-	NA	-
Brain	+	-	NA	-
Diaphragm	++	-	NA	-
Bone marrow	+	-	NA	-
Tumor 2	+			

[1]A scale of - to +++ was used to grade staining.
[2]Mouse 1 had two tumors excised on day of euthanasia: pre and post phage injection.
[3]Not available, mouse died while still anesthetized following tumor 2 excision. No tissues were harvested for IHC.
[4]Control mouse, no phage injected.
[5]Tumor 1 was harvested before phage injected on day of euthanasia. All other tissues harvested after phage injection to serve as positive controls.

Study IV IHC

3 Weeks following final surgery unless otherwise noted.						
Primary antibody=Rabbit α-M13 at 1:10,000 dilution (Sigma cat #B7786, lot#038H4885)						
Staining scale is negative to +++						
			Phage			
Mouse	Harvested	Strain	Injection	Tissue	Comments	Sex
Control[1]	10-14-99	MRL/MpJ-fas$_{LPR}$	no injection	Brain	negative staining, no immunoreactivity	female
				Diaphragm	negative staining, no immunoreactivity	
				Heart	negative staining, no immunoreactivity	
				Kidney	negative staining, no immunoreactivity	
				Lung	negative staining, no immunoreactivity	
				Marrow	negative staining, no immunoreactivity	
				L. Node	negative staining, no immunoreactivity	
				Spleen	negative staining, no immunoreactivity	
				Uterus	negative staining, no immunoreactivity	
				Liver	negative staining, no immunoreactivity	
				Tumor	negative staining, no immunoreactivity	
1	10-15-99	MRL/MpJ-fas$_{LPR}$	ϕ naive 9/21/99	Brain	**+/++ diffuse endothelial reactivity**	female
			ϕ Amp1x 9/21/99	Diaphragm	**++ diffuse endothelial reactivity**	
			ϕ Amp2x 9/24/99	Heart	**+ diffuse endothelial reactivity**	
			ϕ Amp 10/15/99	Kidney	**+ diffuse endothelial reactivity**	
				Lung	**++ but variable reactivity**	
				Marrow	**+ diffuse endothelial reactivity**	
				L. Node	**+/++ staining in endothelial cell, focal reactivity in sinus histiocytes**	
				Spleen	**+ diffuse endothelial reactivity**	
				Uterus	**++ endothelial, red blood cell and serum staining**	
				Liver	**++ endothelial cell and serum staining**	
				Tumor a[2]	negative staining, no immunoreactivity	
				Tumor b	**vascular tumor emboli identifited, tumor cells within vessels show membrane reactivity**	
2	02/24/00	MRL/MpJ-fas$_{LPR}$	ϕ naive 1/31/00	Brain	negative staining, no immunoreactivity	female
			ϕ Amp1x 2/2/00	Diaphragm	negative staining, no immunoreactivity	
			ϕ Amp2x 2/4/00	Heart	negative staining, no immunoreactivity	
				Kidney	negative staining, no immunoreactivity	
				Lung	negative staining, no immunoreactivity	
				Marrow	negative staining, no immunoreactivity	
				L. Node/tumor	negative staining, no immunoreactivity	
				Spleen	negative staining, no immunoreactivity	
				Uterus	negative staining, no immunoreactivity	
				Liver	negative staining, no immunoreactivity	
3	04-05-00	MRL/MpJ-fas$_{LPR}$	ϕ naive 4/3/00	Liver[3]		female
			ϕ Amp1x 4/5/00	Kidney[3]		
				Spleen[3]		
				Lung[3]		
[1]The control mouse did not undergo surgery or phage injection.						
[2]Tumor a was excised before phage injected on 10-15-99.						
All other organs and tumor b were excised following the phage injection on 10-15-99.						
[3]Mouse died following excision of tumor 2 while under halothane anesthesia.						
Only H&E was performed on these tissues						

H&E Results: Study IV

3 Weeks After Three Injections[1]
(In Vivo Screening)

Mouse	1[2]	2	3[3]	Control[4]
Tissue				
Tumor 1	NL[5]	NA[6]	NA[7]	NL
Liver	NL	NL	NL	NL
Spleen	NL	NL	NL	NL
Lymph node	NL	NL	NA[7]	NL
Uterus	NL	NL	NA[7]	NL
Heart	NL	NL	NA[7]	NL
Lung	NL	NL	NL	NL
Kidney	NL	NL	NL	NL
Brain	NL	NL	NA[7]	NL
Diaphragm	NL	NL	NA[7]	NL
Bone marrow	NA[8]	NL	NA[7]	NL
Tumor 2	NL	NA[6]	NA[6]	NA[6]

[1]NL denotes normal histology for the strain. See explanation in Integrated Summary.
[2]Mouse 1 had two tumors excised on the day of euthanasia: pre and post peptide-phage injection.
[3]Mouse died while still anesthetized following tumor excision.
[4]Control mouse, no phage was ever injected.
[5]Tumor 1 was harvested before phage injected on day of euthanasia. All other tissues harvested after phage injection to serve as positive controls.
[6]Not available. Not collected.
[7]Not available. Only liver, kidney, spleen and lung were harvested for H&E.
[8]Not available. Experimental error.

Study IV H&E

3 Weeks following final surgery unless otherwise noted.							
			Phage				
Mouse	Harvested	Strain	Injection	Tissue	Pathology	Comments	Sex
Control[1]	10-14-99	MRL/MpJ-fas$_{LPR}$	no treatment	Brain	normal		female
				Diaphragm	normal		
				Heart	normal		
				Kidney	normal	glomerulo nephritis, interstitial nephritis with focal segmental glomerulo nephritis; moderate sclerosis	
				Lung	normal		
				Marrow	normal		
				L. Node	normal	enlarged node	
				Spleen	normal	enlarged spleen; markedly expanded white pulp with lympho proliferative disorder; extramedulary hematopoiesis	
				Uterus	normal		
				Liver	normal		
				Tumor	normal		
1	10-15-99	MRL/MpJ-fas$_{LPR}$	ϕ naive 9/21/99	Brain	normal		female
			ϕ Amp1x 9/21/99	Diaphragm	normal		
			ϕ Amp2x 9/24/99	Heart	normal		
			ϕ Amp 10/15/99	Kidney	normal	chronic interstitial nephritis; more severe sclerosis than control mouse	
				Lung	normal	peri bronchial lymphocytic infiltrates; probably lympho proliferative disease	
				Marrow	not available	only muscle obtained	
				L. Node	normal		
				Spleen	normal	same as control mouse	
				Uterus	normal		
				Liver	normal		
				Tumor a[2]	normal	looks like normal node	
				Tumor b	normal	looks like lympho proliferative tumor	
2	02/24/00	MRL/MpJ-fas$_{LPR}$	ϕ naive 1/31/00	Brain	normal		female
			ϕ Amp1x 2/2/00	Diaphragm	normal		
			ϕ Amp2x 2/4/00	Heart	normal		
				Kidney	normal	chronic interstitial inflammation and glomerular sclerosis	
				Lung	normal	mild chronic bronchitis	
				Marrow	normal		
				L. Node/tumor	normal		
				Spleen	normal		
				Uterus	normal		
				Liver	normal		
3	04-05-00	MRL/MpJ-fas$_{LPR}$	ϕ naive 4/3/00	Liver[3]	normal	mild portal lymphocytic infiltrates, no hepatic necrosis	female
			ϕ Amp1x 4/5/00	Kidney[3]	normal	interstitial lympoid infiltrates (interstitial nephritis), focal segmental glomerular nephritis	
				Spleen[3]	normal	lymphoid hyperplasia	
				Lung[3]	normal	focal peri bronchial lymphoid aggregates	

[1]The control mouse did not undergo surgery or phage injection.

[2]Tumor a was excised before phage were injected on 10-15-99.
All other organs and tumor b were excised following the phage injection on 10-15-99.

[3]Mouse died following excision of tumor 2 while under halothane anesthesia.

GLP compliance

The studies presented in this IND application were not conducted specifically in compliance with GLP. Dr. Anne Pilaro, a member of the FDA, advised us that this is not necessary for phase I trials.

Previous Human Experience

There is no previous human experience, to our knowledge, with IV injection of peptide-phage libraries, or with IV injection of filamentous phage. There has been extensive human experience with other, related, strains of phage, by Ochs et al and Slopek et al, for example, as discussed in detail in Section 7.

Published material that is relevant to the *safety* of the proposed investigation, which is supplied in full in this application (see Appendix) include:

Ochs' IND
Slopek et al (1983 and 1987)
Yip et al (1999). This paper reports basic pharmacokinetic data of filamentous phage injected IV into mice. The study was not designed to address safety issues- its purpose was to provide information useful in the design of in vivo screening experiments.

Published material that is relevant to an assessment of the *effectiveness* of in vivo screening, which is supplied in full in this application (see Appendix) include:

Arap et al
Rajotte et al
Pasqualini et al 1996

All of these reports are from the same group. These elegant studies demonstrate that, using in vivo screening, small specific peptides can be identified that home specifically to different organs. Peptides were also identified that bound specifically to tumors. When conjugated to doxorubicin, these tumor-homing peptide-doxorubicin conjugates acted as extremely effective anti-tumor drugs in a mouse model. The peptide-doxorubicin conjugates eliminated large tumors and allowed excellent animal survival, without the severe drug toxicity caused by doxorubicin in its free form. These exciting results are what prompted us to initiate similar studies in humans.

In addition to the results of Pasqualini et al, we have also accumulated data in our own lab, as yet unpublished, which also suggest that in vivo screening may be an effective method of identifying peptides which home specifically to tumors, and may ultimately be used to develop effective cancer therapeutics. Our in vivo screening experiments have identified several consensus sequences from peptide-phage eluted from mouse tumors, which suggests that certain peptide-phage clones are binding at least specifically to mouse tissue, and perhaps, specifically to tumor tissue or tumor-associated molecules. An example of one consensus sequence family is included on the next page. Interestingly, these peptides have high amino acid homology with a peptide recently identified as a matrix metalloprotease (MMP) inhibitor. MMPs probably play a major role in pathology caused by tumors- especially tumor invasion and metastasis. The MMP inhibitor, identified by the same group which pioneered in vivo screening (interestingly, the group performed conventional phage-display screening with purified MMP for these experiments, seemingly unrelated to their in vivo screening work), caused significant inhibition of tumor growth and invasion and increased survival in mice with tumors. The MMP inhibitor was specific for forms of MMP found specifically associated with tumors. We will begin a collaboration with Lew Cantley's lab at Harvard soon to test the peptides we identified for MMP binding.

Neither Pasqualini's group or our group have performed in vivo panning in humans. All in vivo screenings have been performed in mice.

In vivo screening of mice with tumors:

Identification of consensus peptides which are highly homologous to matrix metalloprotease inhibitors identified by in vitro phage-display with purified MMP

- **MMP is a promising tumor target, associated with a malignant phenotype**

C E N F **V** G **R** N V E C C R D L **V** **W** **R** **P** Q A C C N M K **V** **W** A T **G** K C **C** W R **H** **W** V S N Y D C **C** **T** G **H** **W** **G** I **G** E N C	Binders identified by our lab by in vivo screening with a mouse with tumors.

C **T** T **H** **W** **G** **F** T L C **C** R R **H** **W** **G** **F** E F C **C** S L **H** **W** **G** **F** W W C	MMP binders, Koivunen et al, *Nature Biotech*, vol 17, Aug 99.

Appendix

1. Abbreviations
2. Literature Cited
3. Protocols and Reagents
    - Phage Amplification and Harvesting Protocol
    - Endotoxin Purification and Removal Prior to In Vivo Injection
    - Reagents Used in Peptide-Phage Production
    - Survival Surgery Protocol
    - Phage Titering Protocol for In Vivo Screenings
    - Phage Titering Protocol for Harvested Organs
2. Papers:
    - Arap et al (1998)
    - Pasqualini et al (1996)
    - Rajotte et al (1998)
    - Slopek et al (1983)
    - Slopek et al (1987)
    - Yip et al (1999)
3. Dr. Hans Ochs materials:
    - Letter of Support
    - BB-IND
    - Information for Investigators

Abbreviations:

ϕ	Phage
ϕX174	A strain of *E. coli* bacteriophage used by Dr. Ochs
DNA	Deoxyribonucleic acid
DPBS	Dulbecco's phosphate buffered saline
EPI	Eukaryotic protease inhibitors
EU	Endotoxin units
FAHC	Fletcher Allen Health Care
fd	Strain of filamentous phage
FDA	Food and Drug Administration
H&E	Hematoxylin and eosin
IHC	Immunohistochemistry
IV	Intravenous
Kan	Kanamycin
LAL	Limulus Amebocyte Lysate
LB	Luria-Bertani
NA	Not available
ND	Not done
NL	Normal
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PI	Principal investigator
pIII	A minor coat protein of filamentous phage
PPI	Prokaryotic protease inhibitors
RPL	Random peptide library
Tet	Tetracycline
TU	Transducing unit

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Phage Amplification and Harvesting Protocol

Day 1:

- Make fresh tetracycline (Tet) stock.
- Prepare 5 pans 2xYT KanTet.
- 3-4PM: streak K91 Kan *E. coli* onto 2xYTKan plate; incubate 37°C overnight.

Day 2:

Prepare (50ml) ready cells:

- Add 50ul Kan (Kanamycin) stock to 50ml 2xYT (2xYT Kan) media in a tube-flask.
- Inoculate with 1 colony from a fresh K91Kan streak.
- Shake 270rpm, 37°C for 5 minutes to mix suspension completely.
- Split the 50 ml cell suspension 2x25ml into 2 tube-flasks.
- Shake 270rpm, 37°C, ~4-5hr.
- Check OD_{600} frequently; do not exceed OD_{600}=0.95.
- At OD_{600}=0.95 shake at 70rpm, 10min.
- Place cells on ice up to 1hr; do not use after 1hr.

Infect library:

- Pool ready cells (50ml total) into one 250 ml tube-flask.
- Under hood with unopened, *sterile*, box of tips, add 40ul each T1-3 and T4 ϕ libraries (gently vortex and spin tubes containing library ϕ before removing the 40ul). Keep sterile! Library is precious!
- Shake slowly, 70rpm, 10min, 37°C.
- Add 2ul Tet stock.
- Shake at 270rpm, 25min.
- Split into 2 oakridge tubes; spin at 9500rpm, 10min; discard supernatants.
- Resuspend and pool cells in a total of 5ml 2xYTKanTet media.
- Add 5ul leupeptin and 5ul aprotinin to the cell suspension.
- Spread 1ml to each of five pans under hood.
- Incubate 37°C overnight.

Day 3:

Harvest peptide-phage:

- Harvest each pan with 3 x 10 ml PBS with fresh prokaryotic protease inhibitors (PPI, Sigma P8465) at 1:1000.
- Spin at 8000 rpm (7649 x g) for 10 min at 4°C.
- Spin the supernatant (supt) at 8000 rpm for 10 min at 4°C. Phage are in the supt.
- Filter supt through 500 ml capacity 0.2 µm PES (polyethylene sulfone) filter unit.
- Precipitate the phage in the filtrate with 0.15 ml cold PEG/ml filtrate. Invert 50x. (PEG=polyethylene glycol)
- Incubate on ice 30 min.
- Centrifuge 9000 rpm (9681 x g) for 20 min at 4° C.
- Resuspend the pellet (phage) in 3-10 ml PBS-PPI.
- Filter through 0.45 µm CA syringe filters into two 15 cc orange capped centrifuge tubes.
- Phage is now ready for the endotoxin removal procedure.

Endotoxin Purification and Removal Prior to In Vivo Phage Injection

1. Turn on waterbath to 37° C.
2. Thaw one vial prokaryotic protease inhibitors cocktail (PPI, Sigma P8465). Need 1 µl PPI/ml DPBS. Prepare DPBS with PPI: 15 ml DPBS (Sigma D8537, endotoxin tested) + 15 µl PPI. (see reagents in Appendix)
3. Place phage into clean 15 cc poly propylene centrifuge tube. Add enough DPBS-PPI to make 10 ml. Vortex.
4. Bring solution to 1% Triton X-114 by adding 100 µl Triton X-114. (Use wide bore tip, drop Triton X directly into solution without placing pipet shaft into tube.) Mix well.
5. Nutate at 4° C for 30 min.
6. Incubate 10' @ 37°C in waterbath.
7. Centrifuge 10', setting #6 (475 x g), Fisher Centrific tabletop centrifuge.
8. Two phases should be visible. Transfer top phase, getting as close to miniscus as possible, to clean 15 cc tube.
9. Repeat steps 4-8 twice more. When transferring top phase after final centrifugation, transfer to Oakridge tube, measuring volume.
10. Add 0.15 ml cold PEG (polyethylene glycol)/ml phage solution to precipitate phage. Mix well by swirling 50x. Incubate on ice 30'.
11. Centrifuge in Sorvall Super T @ 9000rpm (9681 x g) for 20-30'.
12. Place tube in slant rack for transport. Aspirate supernatant. Phage are in pellet. Allow pellet to drain an additional 5' and aspirate any additional supernatant.
13. Resuspend pellet with 1000 µl endotoxin tested DPBS with eukaryotic protease inhibitors (EPI, Sigma P8340) at 1:1000.
14. Shake @ 200 rpm for 10' on ice.
15. Transfer phage solution to 1.5 ml screw cap Eppendorf tube(s).
16. Rinse Oakridge tube with 2 x 500 µl DPBS-EPI and add to Eppendorfs.
17. Centrifuge in Heraeus 5' @ 13000 rpm (13,800 x g). Should see a small pellet. <u>Phage is still in supernatant.</u>
18. Filter supernatant through 0.45 µm cellulose acetate membrane (Schleicher + Schuell, #10465202, cellulose acetate filter) using 3 cc luer lock syringe.
19. Filter through 0.2 µm cellulose acetate membrane (Schleicher + Schuell, #10465200) as above.
20. Bring to desired volume using DPBS-EPI. Aliquot into sterile tubes. Save some for "input" titering.

Reagents used in peptide-phage production

2xYT Media

16.0 g/l Tryptone
10.0 g/l Yeast Extract
5.0 g/l Sodium Chloride
15.0 g/l Agar
240 µL/l Kanamycin (100mg/mL ddH2O (w,v))
4.0 mL/l Tetracycline (5mg/mL Ethanol (w,v)
Autoclave. At 50° C add antibiotics. Pour 250mL / pan

DPBS (Dulbecco's Phosphate Buffered Saline), pH 7.3

Sigma Product Number: D 8537
0.2 g/l Potassium chloride
0.2 g/l Postassium Phosphate Monobasic (anhydrous)
8.0 g/l Sodium Chloride
1.15 g/l Sodium Phosphate Dibasic (anhydrous)
Sterile filtered and endotoxin tested

EPI (eukaryotic protease inhibitors)

Sigma Product Number: P 8340
Inhibitors Present:
4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF)
pepstatin A
trans- epoxysuccinyl-L-leucylamido (4-guanidino) butane (E-64)
bestatin
leupeptin
aprotinin

PBS (Phosphate Buffered Saline), pH 7.3

1.9mM Sodium Phosphate Monobasic (anhydrous)
8.1mM Sodium Phosphate Dibasic (anhydrous)
154mM NaCl

PEG/NaCl

20% Polyethylene Glycol (w,v)
2.5M Sodium Chloride

PPI (prokaryotic protease inhibitors)

Sigma Product Number: P 8465
Inhibitors Present:
4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF)
pepstatin A
trans- epoxysuccinyl-L-leucylamido (4-guanidino) butane (E-64)
bestatin
sodium EDTA

Triton X-114 (Octylphenoxypoly-ethoxyethanol)

Sigma Product Number X 114

Survival Surgery Protocol

1. Weigh the mouse prior to procedure.
2. Position a warming pad beneath the mouse during the procedure to maintain body temperature.
3. Anesthetize the mouse using halothane, to effect.
4. Apply opthamic ointment to the eyes of the mouse.
5. When the mouse is unresponsive to toe pinch:
   a.) Apply a warm compress to the tail to dilate the tail vein.
   b.) Using a 29 gauge needle, inject 250 µl or less of sterile peptide-phage preparation into the tail vein.
   c.) Allow the material to circulate for 10 minutes.
4. Using clippers, shave the area immediately surrounding the tumor to be excised.
5. Wipe the shaved area with alcohol and sterile drape.
6. At the end of the 10 minute period, using aseptic technique, excise the subcutaneous tumor of interest through a small skin incision.
7. Note the tumor weight and place on ice.
8. Close the incision using interrupted 5-0 nylon with a PC-1 cutting needle.
9. Inject the mouse subcutaneously with ½ dose of buprenorphine (0.05 mg/kg) for pain. (The other ½ to be administered in 12 hrs.)
10. Turn off the halothane. Allow the animal to breath pure O2 until it is responsive.
11. Place the mouse in its cage and position a heat lamp over the mouse during the recovery period. Place drapes over the cage to protect the animal's eyes from the light.
12. Observe the animal for 2 hours, or until the animal has fully recovered. Return the animal to the animal care facility.

Phage Titering Protocol for In Vivo Screenings

1. Prepare dilutions (using PBS) of the phage to be titered.
2. Place 10 µl of each phage dilution into the bottow of a 17 x 100 mm polypropylene round-bottomed tube.
3. Add 10 µl of "ready cells."
   Ready cells=K91 kanamycin resistant *E. coli* grown in Kan-supplemented terrific broth until OD_{600}=0.3.
4. Incubate for 10 minutes at room temperature.
5. Add 1.0 ml Luria-Bertani media containing 0.2 µg/ml tetracycline (LB-tet).
6. Incubate at 37° C, with shaking at 260 rpm, for 25 minutes.
7. Incubate on ice for 10 minutes.
8. Plate 20 µl of the suspension onto LB kanamycin/tetracycline supplemented media agar.
9. Plate 20 µl of "ready cells" as a negative control.
10. Incubate overnight at 37° C.
11. One colony=one transducing unit(TU).

Phage Titering Protocol for Harvested Organs

1. Place the tissue (or 20 µl heparinized whole blood) in a tared disposable homogenizing (sterile) tube. Record the tissue weight.
2. Add 75 µl of phosphate-buffered saline with eukaryotic protease inhibitors (PBS-EPI) at 1:1000.
3. Homogenize each sample using a sterile disposable pestle inserted in the battery operated homogenizer.
4. Add 75 µl "ready cells."
   Ready cells=K91 kanamycin resistant *E. coli* grown in Kan-supplemented terrific broth until OD_{600}=0.3.
5. Gently mix suspension by flicking the tube.
6. Incubate 1 hour at room temperature, flicking the tube every 15 minutes.
7. Add tetracycline for a final concentration of 0.2 µg/ml.
8. Incubate at 37° C, with shaking at 270 rpm, for 25 minutes.
9. Centrifuge at 9000 rpm (6610 x g) for 5 minutes at 4° C.
10. Remove all but about 50 µl of the supernatant.
11. Resuspend the pellet in the remaining supernatant.
12. Plate the entire suspension onto LB media agar supplemented with kanamycin and tetracycline.
13. Incubate at 37° C overnight.

Cancer Treatment by Targeted Drug Delivery to Tumor Vasculature in a Mouse Model

Wadih Arap,* Renata Pasqualini,* Erkki Ruoslahti†

In vivo selection of phage display libraries was used to isolate peptides that home specifically to tumor blood vessels. When coupled to the anticancer drug doxorubicin, two of these peptides—one containing an α_v integrin–binding Arg-Gly-Asp motif and the other an Asn-Gly-Arg motif—enhanced the efficacy of the drug against human breast cancer xenografts in nude mice and also reduced its toxicity. These results indicate that it may be possible to develop targeted chemotherapy strategies that are based on selective expression of receptors in tumor vasculature.

Endothelial cells in the angiogenic vessels within solid tumors express several proteins that are absent or barely detectable in established blood vessels (*1*), including α_v integrins (*2*) and receptors for certain angiogenic growth factors (*3*). We have applied in vivo selection of phage peptide libraries to identify peptides that home selectively to the vasculature of specific organs (*4*, *5*). The results of our studies imply that many tissues have vascular "addresses." To determine whether in vivo selection could be used to target tumor blood vessels, we injected phage peptide libraries into the circulation of nude mice bearing human breast carcinoma xenografts.

Recovery of phage from the tumors led to the identification of three main peptide motifs that targeted the phage into the tumors (*6*). One motif contained the sequence Arg-Gly-Asp (RGD) (*7*, *8*), embedded in a peptide structure that we have shown to bind selectively to $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins (*9*). Phage carrying this motif, CDCRGDCFC (termed RGD-4C), homes to several tumor types (including carcinoma, sarcoma, and melanoma) in a highly selective manner, and homing is specifically inhibited by the cognate peptide (*10*).

A second peptide motif that accumulated in tumors was derived from a library with the general structure $CX_3CX_3CX_3C$ (X = variable residue, C = cysteine) (*6*). This peptide, CNGRCVSGCAGRC, contained the sequence Asn-Gly-Arg (NGR), which has been identified as a cell adhesion motif (*11*). We tested two other peptides that contain the NGR motif but are otherwise different from CNGRCVSGCAGRC: a linear peptide, NGRAHA (*11*), and a cyclic peptide, CVLNGRMEC. Tumor homing for all three peptides was independent of the tumor type and species; the phage homed to a human breast carcinoma (Fig. 1A), a human Kaposi's sarcoma, and a mouse melanoma (*12*). We synthesized the minimal cyclic NGR peptide from the CNGRCVSGCAGRC phage and found that this peptide (CNGRC), when coinjected with the phage, inhibited the accumulation of the CNGRCVSGCAGRC phage (Fig. 1A) and of the two other NGR-displaying phages in breast carcinoma xenografts (*12*).

The third motif—Gly-Ser-Leu (GSL) and its permutations—was frequently recovered from screenings using breast carcinoma (*6*), Kaposi's sarcoma, and malignant melanoma, and homing of the phage was inhibited by the cognate peptide (Fig. 1B). This motif was not studied further here.

The RGD-4C phage homes selectively to breast cancer xenografts (Fig. 1C). This homing can be inhibited by the free RGD-4C peptide (*10*), but not by the CNGRC peptide, even when this peptide was used in amounts 10 times those that inhibited the homing of the NGR phage (Fig. 1D). Tumor homing of the NGR phage was also partially inhibited by the RGD-4C peptide (Fig. 1E), but this peptide was only 10 to 20% as potent as CNGRC. An unrelated cyclic peptide, GACVFSIAHECGA, had no effect on the tumor-homing ability of either phage (*12*). Thus, our in vivo screenings yielded two peptide motifs, RGD-4C and NGR, both of which had previously been reported

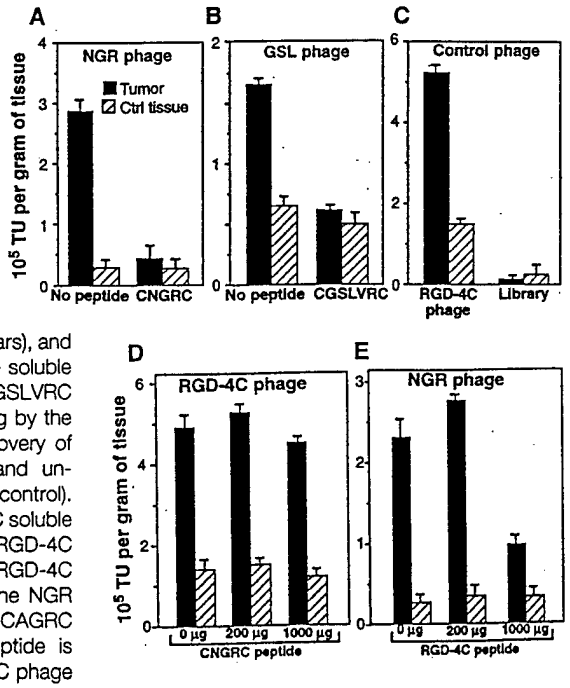

Fig. 1. Recovery of phage displaying tumor-homing peptides from breast carcinoma xenografts. Phage [10^9 transducing units (TU)] was injected into the tail vein of mice bearing size-matched MDA-MB-435–derived tumors (~1 cm^3) and recovered after perfusion. Mean values for phage recovered from the tumor or control tissue (brain) and the SEM from triplicate platings are shown. (**A**) Recovery of CNGRCVSGCAGRC phage from tumor (solid bars) and brain (striped bars), and inhibition of the tumor homing by the soluble peptide CNGRC. (**B**) Recovery of CGSLVRC phage and inhibition of tumor homing by the soluble peptide CGSLVRC. (**C**) Recovery of RGD-4C phage (positive control) and unselected phage library mix (negative control). (**D**) Increasing amounts of the CNGRC soluble peptide were injected with the RGD-4C phage. (**E**) Increasing amounts of the RGD-4C soluble peptide were injected with the NGR phage. Inhibition of the CNGRCVSGCAGRC phage homing by the CNGRC peptide is shown in (A); inhibition of the RGD-4C phage by the RGD-4C peptide has been reported (*10*).

Cancer Research Center, The Burnham Institute, 10901 North Torrey Pines Road, La Jolla, CA 92037, USA.

*These authors contributed equally to this report.
†To whom correspondence should be addressed. E-mail: ruoslahti@burnham-inst.org

to bind to integrins (9, 11). The affinity of NGR for integrins is about three orders of magnitude less than that of RGD peptides (7, 11). Nevertheless, the homing ratio (tumor/control organ) of the phage displaying the NGR motif was three times that of the RGD-4C phage (12). This discrepancy in activities, and the cross-inhibition results described above, strongly suggest that the NGR and RGD-4C peptides bind to different receptors in the tumors.

We next studied phage homing to tumors by immunostaining (Fig. 2). In one set of experiments (13), phage was allowed to circulate for 3 to 5 min, followed by perfusion (10) and immediate tissue recovery. In the second set, tissues were analyzed 24 hours after phage injection, when there is almost no phage left in the circulation (10). Strong phage staining in tumor vasculature, but not in normal endothelia, was seen in the short-term experiments with CNGRCVSGCAGRC phage in MDA-MB-435 cell–derived human breast carcinoma xenografts (Fig. 2A) and SLK cell–derived human Kaposi's sarcoma xenografts (Fig. 2B). The two other NGR phages, NGRAHA and CVLNGRMEC, also showed strong tumor staining (12), whereas a control phage showed no staining (Fig. 2, E and F). At 24 hours, the staining pattern indicated that the NGR phage had spread outside the blood vessels and into the tumors (Fig. 2, C and D). This spreading may be attributable to increased permeability of tumor blood vessels (14) or uptake of the phage by angiogenic endothelial cells (15) and subsequent transfer to tumor tissue.

The CNGRCVSGCAGRC phage showed the greatest tumor selectivity among all the peptides analyzed. Several control organs showed very low or no immunostaining, confirming the specificity of the NGR motif for tumor vessels; heart (Fig. 2G) and mammary gland (Fig. 2H) are shown (16). Spleen and liver, which are part of the reticuloendothelial system (RES), contained phage; uptake by the RES is a general property of the phage particle and is independent of the peptide it displays (10, 17). These immunostaining results with the NGR phage are similar to observations made with the RGD-4C phage (10).

To determine whether the tumor-homing peptides RGD-4C and CNGRC could be used to improve the therapeutic index of cancer chemotherapeutics, we coupled them to doxorubicin (dox) (18). Dox is one of the most frequently used anticancer drugs and one of a few chemotherapeutic agents known to have antiangiogenic activity (19). The dox-peptide conjugates were used to treat mice bearing tumors derived from human MDA-MB-435 breast carcinoma cells.

The commonly used dose of dox in nude mice with human tumor xenografts is 50 to 200 μg/week (20). Because we expected the dox conjugates to be more effective than the free drug, we initially used the conjugates at a dose of dox-equivalent of only 5 μg/week (13, 21). Tumor-bearing mice treated with RGD-4C conjugate outlived the control mice, all of which died from widespread disease (Log-Rank test, $P < 0.0001$; Wilcoxon test, $P = 0.0007$) (Fig. 3A). In a dose-escalation experiment, tumor-bearing mice were treated with the dox-RGD-4C conjugate at 30 μg of dox-equivalent every 21 days for 84 days and were then observed, without further treatment, for an extended period of time. All of these mice outlived the dox-treated mice by more than 6 months, suggesting that both primary tumor growth and metastasis were inhibited by the conjugate. Many of the tumors in the mice that received the dox-RGD-4C conjugate (30 μg of dox-equivalent every 21 days) showed marked skin ulcer-

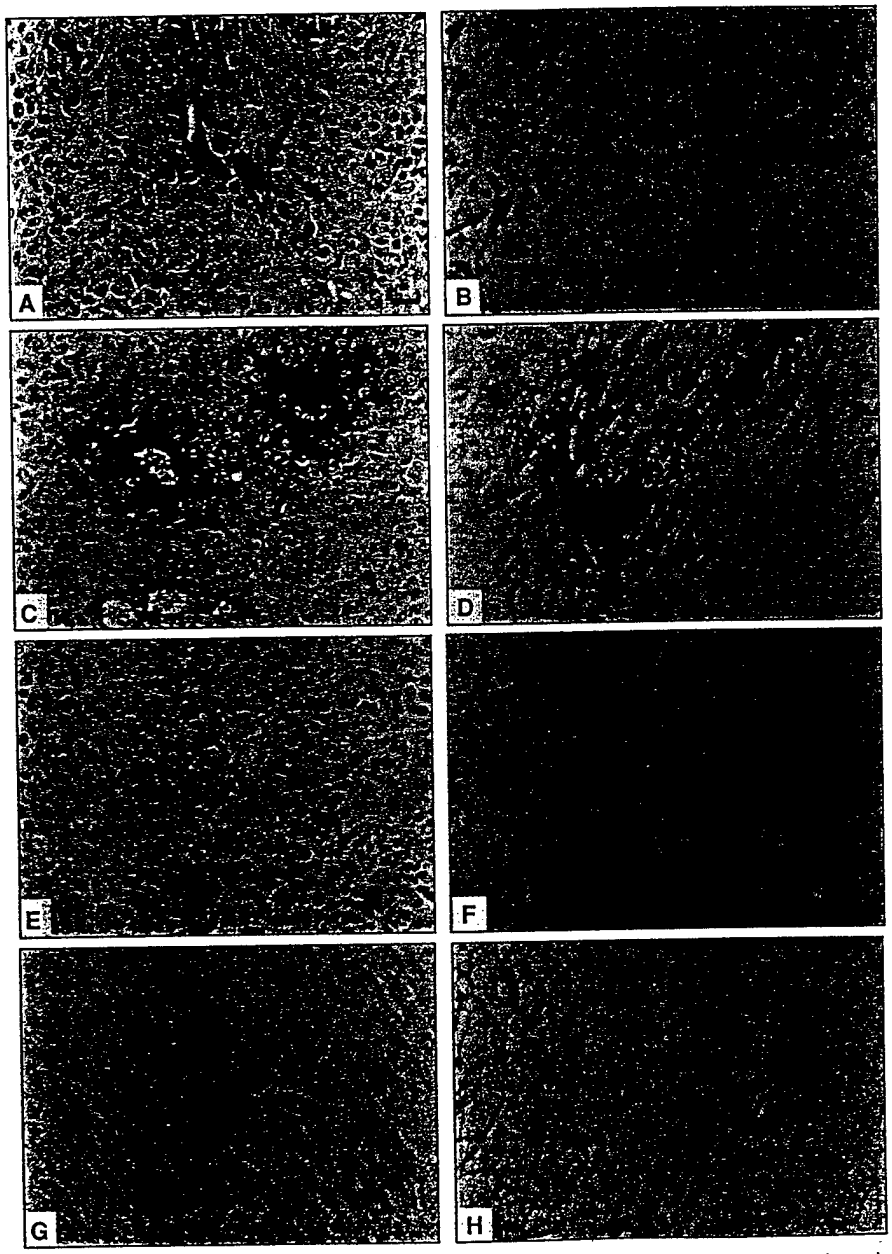

Fig. 2. Immunohistochemical staining of phage after intravenous injection into tumor-bearing mice. Phage displaying the peptide CNGRCVSGCAGRC (**A** to **D**, **G**, and **H**) or control phage with no insert (**E** and **F**) were injected intravenously into mice bearing MDA-MB-435–derived breast carcinoma (A, C, and E) and SLK-derived Kaposi's sarcoma (B, D, and F) xenografts. Phage was allowed to circulate for 4 min (A, B, E, and F) or for 24 hours (C, D, G, and H). Tumors and control organs were removed, fixed in Bouin solution, and embedded in paraffin for preparation of tissue sections. An antibody to M-13 phage (Pharmacia) was used for the staining. Heart (G) and mammary gland (H) are shown as control organs (16). Arrows point to blood vessels. Scale bar in (A), 5 μm.

ation and tumor necrosis, whereas these signs were not observed in any of the control groups. At necropsy, the mice treated with the dox-RGD-4C conjugate had significantly smaller tumors (*t* test, $P = 0.02$), less spreading to regional lymph nodes ($P < 0.0001$), and fewer pulmonary metastases ($P < 0.0001$) than did the mice treated with free dox (Fig. 3, B to D). Similar results were obtained in five independent experiments. Histopathological analysis revealed pronounced destruction of the tumor architecture and widespread cell death in the tumors of mice treated with the dox-RGD-4C conjugate; tumors treated with free dox at this dose were only minimally affected. In contrast, the dox-RGD-4C conjugate was less toxic to the liver and heart than was free dox (Fig. 3E). In some experiments, dox together with unconjugated soluble peptide was used as a control; the drug-peptide combination was no more effective than free dox (*12*).

To assess toxicity, we used 200 μg of dox-equivalent in mice with large (~5 cm^3), size-matched tumors (*13*, *21*). Mice treated with the dox-RGD-4C conjugate survived more than a week, whereas all of the dox-treated mice died within 48 hours of drug administration (Fig. 3F). Accumulation of dox-RGD-4C within the large tumors thus appeared to have sequestered the conjugated drug, thereby reducing its toxicity to other tissues.

Less extensive data with the CNGRC peptide conjugate indicated an efficacy similar to that of the RGD-4C conjugate. In all experiments, tumors treated with the dox-CNGRC conjugate were one-fourth to one-fifth as large as tumors treated in the control groups (Fig. 4A). A marked reduction in metastasis and a prolongation of long-term survival were also seen (Log-Rank test, $P = 0.0064$; Wilcoxon test, $P = 0.0343$) (Fig. 4B). Two of the six dox-CNGRC–treated animals were still alive more than 11 weeks after the last of the control mice died. The dox-CNGRC conjugate was also less toxic than the free drug (Fig. 4C). CNGRC-peptide alone failed to reproduce the effect of the conjugate, even in doses up to 150 μg/week. Unconjugated CNGRC-dox mixture

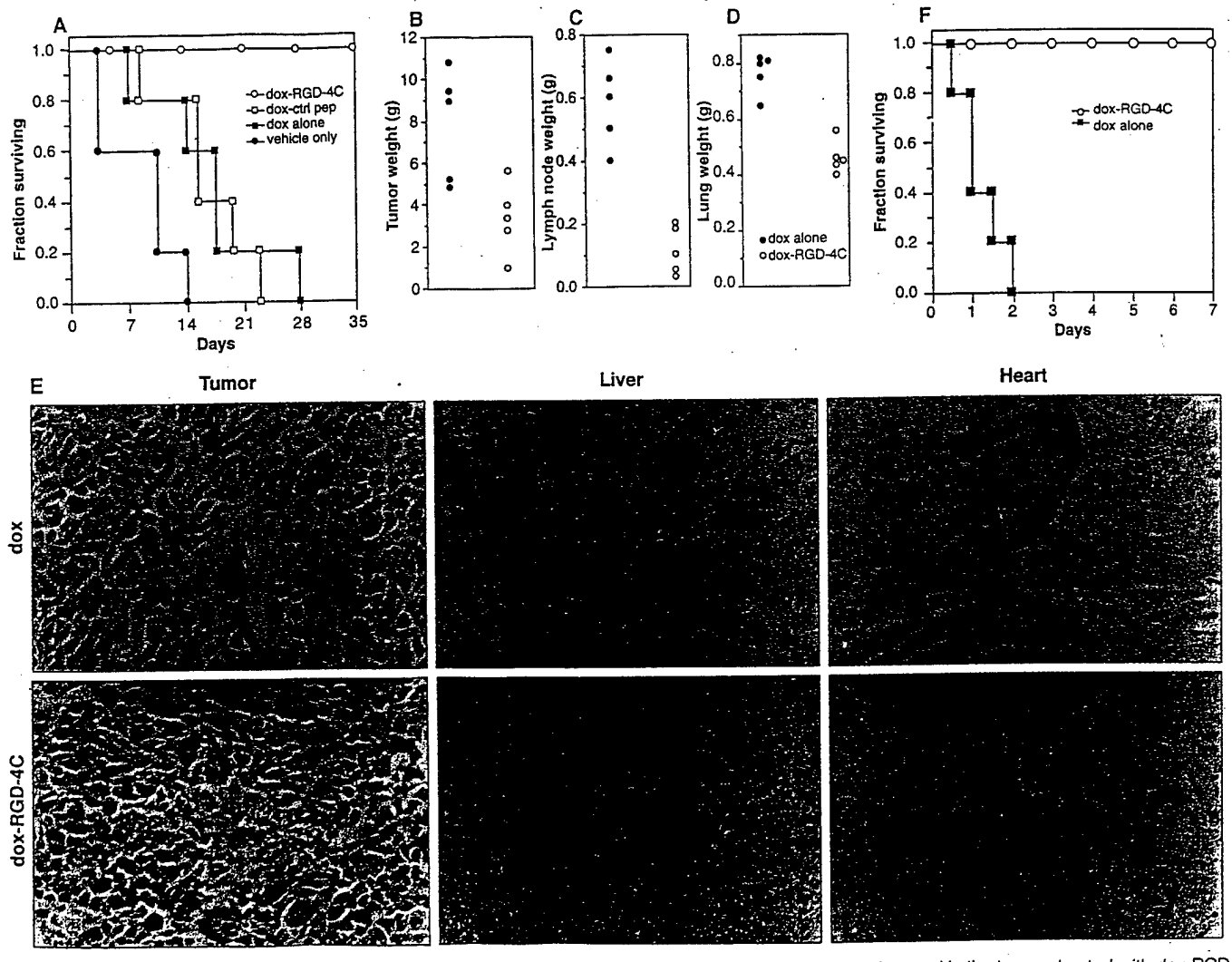

Fig. 3. Treatment of mice bearing MDA-MB-435–derived breast carcinomas with dox-RGD-4C peptide conjugate. Mice with size-matched tumors (~1 cm^3) were randomized into four treatment groups (five animals per group): vehicle only, free dox, dox-control peptide (GACVFSIAHECGA; dox-ctrl pep), and dox-RGD-4C conjugate. (**A**) Mice were treated with 5 μg/week of dox-equivalent. A Kaplan-Meier survival curve is shown. (**B** to **D**) Mice were treated with 30 μg of dox-equivalent every 21 days. The animals were killed, and tumors (B), axillary lymph nodes (C), and lungs (D) were weighed after three treatments. (**E**) Histopathological analysis (hematoxylin and eosin stain) of MDA-MB-435 tumors, liver, and heart treated with dox or dox-RGD-4C conjugate. Vascular damage was observed in the tumors treated with dox-RGD-4C conjugate (arrows, lower left panel), but not in the tumors treated with free dox (arrows, upper left panel). Signs of toxicity were seen in the liver and heart of mice treated with dox (arrows, upper middle and upper right panels), whereas the blood vessels were relatively undamaged in the mice treated with the dox-RGD-4C conjugate. The changes were scored blindly by a pathologist; representative micrographs are shown. Scale bar, 7.5 μm. (**F**) Mice bearing large (~5 cm^3) MDA-MB-435 breast carcinomas (four animals per group) were randomized to receive a single dose of free dox or dox-RGD-4C conjugate at 200 μg of dox-equivalent per mouse. A Kaplan-Meier survival curve is shown.

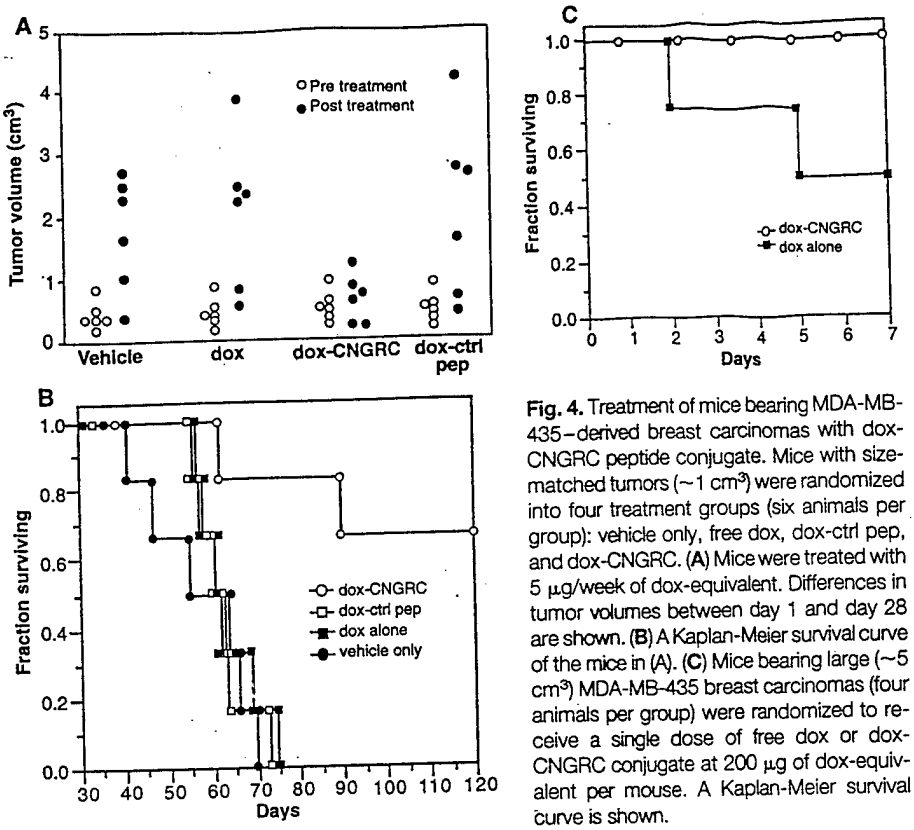

Fig. 4. Treatment of mice bearing MDA-MB-435–derived breast carcinomas with dox-CNGRC peptide conjugate. Mice with size-matched tumors (~1 cm^3) were randomized into four treatment groups (six animals per group): vehicle only, free dox, dox-ctrl pep, and dox-CNGRC. (**A**) Mice were treated with 5 μg/week of dox-equivalent. Differences in tumor volumes between day 1 and day 28 are shown. (**B**) A Kaplan-Meier survival curve of the mice in (A). (**C**) Mice bearing large (~5 cm^3) MDA-MB-435 breast carcinomas (four animals per group) were randomized to receive a single dose of free dox or dox-CNGRC conjugate at 200 μg of dox-equivalent per mouse. A Kaplan-Meier survival curve is shown.

was no different from dox alone. The dox-CNGRC conjugates were also effective against xenografts derived from another human breast carcinoma cell line, MDA-MB-231 (*12*).

We expect the NGR and RGD-4C motifs to target human vasculature as well, because (i) the NGR phage binds to blood vessels of human tumors and less so than to vessels in normal tissue (*22*), and (ii) the RGD-4C peptide binds to human α_v integrins (*9*, *10*), which are known to be selectively expressed in human tumor blood vessels (*23*). Thus, these peptides are potentially suitable for tumor targeting in patients. The RGD-4C peptide is likely to carry dox into the tumor vasculature and also to the tumor cells themselves, because the MDA-MB-435 breast carcinoma expresses α_v integrins (*10*). Because many human tumors express the α_v integrins (*23*), our animal model is a reasonable mimic of the situation in at least a subgroup of cancer patients. The targeting of drugs into tumors is a new use of the selective expression of α_v integrins and other receptors in tumor vasculature. The effectiveness of the CNGRC conjugate may be derived entirely from vascular targeting because the NGR peptides do not bind to the MDA-MD-435 cells (*12*).

The tumor vasculature is a particularly suitable target for cancer therapy because it is composed of nonmalignant endothelial cells that are genetically stable and therefore unlikely to mutate into drug-resistant variants (*24*). In addition, these cells are more accessible to drugs and have an intrinsic amplification mechanism; it has been estimated that elimination of a single endothelial cell can inhibit the growth of 100 tumor cells (*24*). New targeting strategies, including the ones described here, have the potential to markedly improve cancer treatment.

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involving this motif, leading to a zipper-like model for cadherin-mediated cell adhesion[10]. These antiparallel 'adhesive interactions' are not seen in the Ecad12 crystal structure, although sterically Ecad12 could be accommodated in the zipper-like arrangement. Regardless, we have shown that the building block of any higher-order organization is a parallel cadherin dimer whose structure is promoted by and dependent on the presence of bound calcium ions. The dimerization, rigidification and resulting mechanical stabilization of E-cadherin explains, at least in part, the calcium requirement for the integrity of cell junctions[19]. □

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CORRESPONDENCE and requests for materials to be addressed to J.M.R. (e-mail rini@gene4d.med.utoronto.ca).

Organ targeting *in vivo* using phage display peptide libraries

Renata Pasqualini & Erkki Ruoslahti

La Jolla Cancer Research Center, The Burnham Institute, 10901 North Torrey Pines Road, La Jolla, California 92037, USA

PREFERENTIAL homing of tumour cells[1,2] and leukocytes[3,4] to specific organs indicates that tissues carry unique marker molecules accessible to circulating cells. Organ-selective address molecules on endothelial surfaces have been identified for lymphocyte homing to various lymphoid organs and to tissues undergoing inflammation[5–8], and an endothelial marker responsible for tumour homing to the lungs has also been identified[9]. Here we report a new approach to studying organ-selective targeting based on *in vivo* screening of random peptide sequences. Peptides capable of mediating selective localization of phage to brain and kidney blood vessels were identified, and showed up to 13-fold selectivity for these organs. One of the peptides displayed by the brain-localizing phage was synthesized and shown to specifically inhibit the localization of the homologous phage into the brain. When coated onto glutaraldehyde-fixed red blood cells, the peptide caused selective localization of intravenously injected cells into the brain. These peptide sequences represent the first step towards identifying selective endothelial markers, which may be useful in targeting cells, drugs and genes into selected tissues.

We injected phage libraries[10–13] intravenously into mice and subsequently rescued the phage from individual organs. Within the time frame of the experiments, the bulk of the phage remained in circulation (not shown). Some organs, such as liver and lung, captured too many phage to be used as target organs for selection; we focused on peptide sequences that directed phage binding to the brain and kidney, because these organs bound relatively few phage from the unselected libraries.

To select peptides that home to the brain, phage were injected intravenously, recovered from the brain, amplified repeatedly *in vitro*, and re-injected to obtain sufficient enrichment. Although more of the injected phage were recovered from an equivalent amount of kidney than from brain after the first injection, 6- and 13-fold more phage were recovered from the brain in the second and third rounds of the selection, respectively. This enrichment for the brain was reproducible in several experiments. Results from representative experiments performed with two different mixtures of libraries are shown in Fig. 1.

Sequencing of the inserts from 48 brain-localizing phage from library pool I revealed three dominant amino-acid sequence motifs. Peptides containing an SRL motif represented 54% of the clones, followed by a CENWWGDVC motif (29%). Other motifs that appeared more than once included CKDWGRIC, CVLRGGRC and CTRITESC. Many of these less common motifs shared the sequence RI/RL with the more common ones. Eight sequences were seen only once and probably represent background. From the library pool II phage, 25 sequences revealed only one motif, WRCVLREGPAGGCAWFNRHRL, which comprised 40% of the sequences.

The SRL tripeptide was found in several sequence contexts, indicating that the sequences were derived from a number of independent phage. Moreover, the DNA sequences of phage displaying the same peptide were in some cases not identical. The strong selection for predominant motifs and their internal diversity clearly shows that the peptide displayed by the phage, rather than some incidental mutant property of the phage, is responsible for the selective binding.

When tested as isolated phage, the CLSSRLDAC, CNSRLHLRC, CENWWGDVC and WRCVLREGPAGGCAWFNRHRL phage each targeted the brain several-fold more effectively than the kidney. The brain/kidney ratios (number of phage recovered from brain divided by number of phage recovered from the same amount of kidney tissue) were about 8 for the CLSSRLDAC and the CNSRLHLRC phage, 4 CENWWGDVC, and 9 for WRCVLREGPAGGCAWFNRHRL. A phage that had not been selected for brain or kidney binding gave a brain/kidney ratio of approximately 1.

Phage that would home selectively into the kidney were isolated from a mixture of the CX_5C and CX_6C libraries. The enrichment was three- to fivefold (data not shown). Phage carrying two motifs, CLPVASC and CGAREMC, constituted 60% of the 48 phage sequenced; CKGRSSAC appeared three times. The preferential binding to kidney relative to brain was highest, about sevenfold, with CLPVASC phage. Control phage again gave a kidney/brain ratio of about 1.

Immunohistochemical staining of the brain-binding phage displaying CLSSRLDAC revealed staining within the brain capil-

laries (Fig. 2*a*). No preference for any part of the brain was seen. Injection of the kidney-binding CLPVASC phage did not cause staining of the brain capillaries (Fig. 2*b*). In contrast, the kidney-binding phage was found in the glomeruli and in between the tubules (Fig. 2*c*). Only slight staining was seen in the kidney with the brain-binding phage (Fig. 2*d*).

A soluble cyclic peptide was synthesized according to one of the brain-binding phage sequences, CLSSRLDAC. We chose to synthesize this peptide because it gave a slightly higher (8.2 versus 7.6) brain/kidney ratio than the most prevalent motif, CNSRLHLRC. The CLSSRLDAC peptide inhibited the preferential localization into the brain of the phage carrying the same

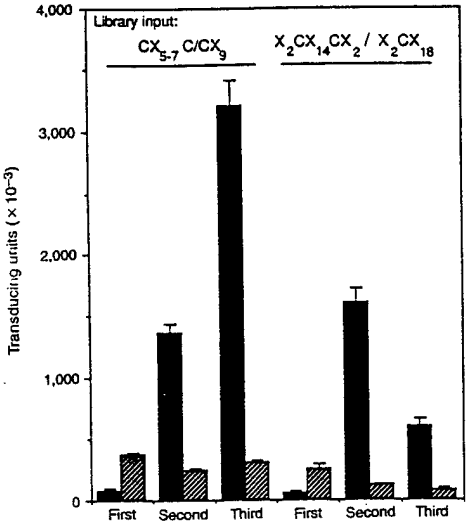

FIG. 1 Selective localization of phage to the brain. Two library pools, $CX_{5-7}C/CX_9$ (pool I)[10–12] and $X_2CX_{14}CX_2$, X_2CX_{18} (pool II) (E. Koivunen and E.R., unpublished data) were injected in the tail vein. After 1–4 min the mice were killed and the phage rescued from tissues. Phage recovered from the brain were amplified and re-injected in two consecutive rounds. The number of phage (transducing units) recovered from brain (black bars) and kidney (hatched bars) tissue in a representative experiment with each library pool is shown. The number of phage recovered from different organs varied to some extent, but the ratios were consistent across experiments. The bars show standard error of the mean (s.e.m.) from plating in triplicate.

METHODS. The libraries were prepared as described[10–13], and display mostly cyclic peptides, which often bind with higher affinity than non-cyclic peptides[11,12]. Balb/c (2-month-old females; Jackson Laboratories, Bar Harbor, ME) were anaesthetized with Avertin (0.015 ml g^{-1}) and injected intravenously (tail vein) with a mixture of phage libraries containing 10^{16} (pool I) and 10^{14} (pool II) transducing units diluted in 200 µl DMEM. At the end of the experiment the mice were snap-frozen in liquid nitrogen, while in a state of deep anaesthesia. To recover the bound phage, the carcasses were partly thawed at room temperature, organs were removed, weighed, and ground in 1 ml DMEM-PI (DMEM containing the protease inhibitors phenyl methyl sulphonyl fluoride (1 mM), aprotinin (20 µg ml^{-1}) and leupeptin (1 µg m^{-1})). The tissue were washed three times with ice-cold DMEM-PI containing 1% BSA and incubated with 1 ml of bacteria for 1 h. NZY medium (10 ml) containing 0.2 µg ml^{-1} tetracycline was added, the mixture was incubated in a 37 °C shaker for 1 h, and 200-µl portions were plated in agar plates in the presence of 40 µg ml^{-1} tetracycline. About 200 individual colonies were grown separately for 16 h in 5 ml NZY medium containing 40 µg ml^{-1} tetracycline. The bacterial cultures were then pooled and the amplified phage were injected into mice as described above.

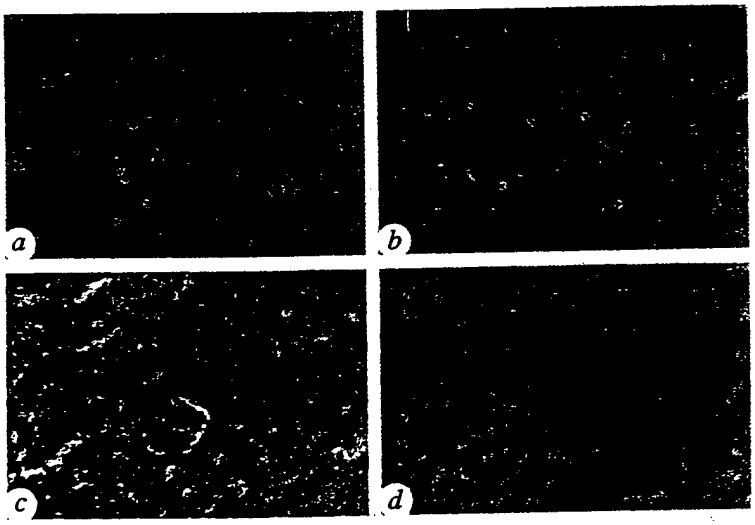

FIG. 2 Immunohistochemical staining of phage in brain and kidney tissue. Phage were amplified individually and injected into mice. Tissue sections were prepared after perfusion of the mice through the heart with DMEM, and the organs were fixed in Bouin solution. An antibody against M13 (Pharmacia Biotech, Piscataway, NJ) was used for the staining, followed by a peroxidase-conjugated secondary antibody (Sigma, St Louis, MO). Brain-selective phage displaying CLSSRLDAC in brain (*a*) and in kidney (*d*), and the kidney-selective phage displaying CLPVASC in kidney (*c*) and in brain (*b*) are shown. Magnification: *a*, ×400; *b–d*, ×200.

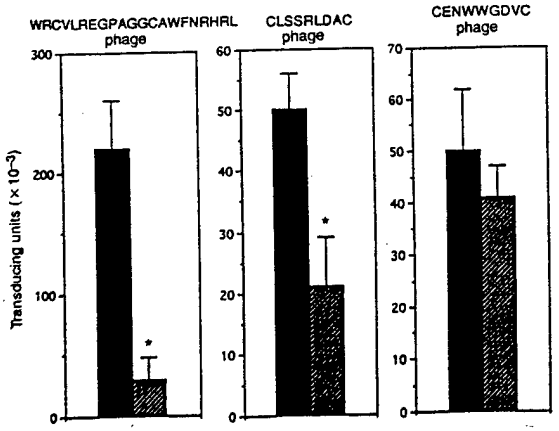

FIG. 3 Effect of the CLSSRLCAC synthetic peptide on the *in vivo* localization of phage. Brain-binding phage displaying CLSSRLDAC, CENWWDGVC and WRCVLREGPAGGCAWFNRHRL motifs were titrated to the same concentration and 10^8 transducing units were injected into mice either on their own (black bars) or together with 500 µg of the CLSSRLDAC synthetic peptide (hatched bars). Shown is the number of phage (transducing units) recovered from the brain. The CLSSRLDAC peptide was synthesized and purified by high-performance liquid chromatography (Immunodynamics, La Jolla, CA). Bars show s.e.m. from triplicates. Asterisks indicate statistically significant differences (unpaired Student *t*-test, $P < 0.05$).

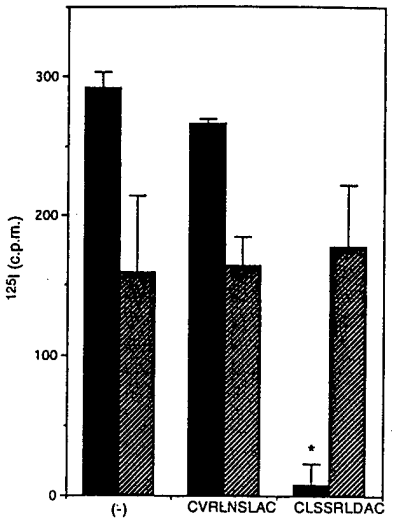

FIG. 4 Tissue localization of red blood cells coated with CLSSRLDAC peptide. Inhibition of brain localization by the corresponding soluble peptide. Iodinated CLSSRLDAC peptide was coupled to red blood cells and injected into the tail vein of mice in the presence or absence of unlabelled peptides. Radioactivity in perfused brain (black bars) and kidney (hatched bars) tissues is shown.

METHODS. The CLSSRLDAC peptide (1 mg) was labelled using the Bolton Hunter reagent (Amersham Life Science, Arlington Heights, IL), and purified by reversed-phase chromatography on Sep-Pak cartridges (Waters, Millipore, Milford, MA). The labelled peptide (100 μg) was coupled to 1 ml glutaraldehyde-stabilized sheep red blood cells (Sigma, St Louis, MO) according to the manufacturer's instructions. The coated cells (50 μl, 200,000 c.p.m.) were injected into the tail vein of mice in the presence or absence of 10 mM of unlabelled CLSSRLDAC peptide. An unrelated peptide, CVRLNSLAC, was used as a control. The mice were killed 2 min later, perfused through the heart with 50 ml DMEM, and their brain and kidneys were removed and assayed for radioactivity. The animals were treated in accordance with the Institute's Animal Facility Guidelines. Bars show s.e.m. from triplicates. Asterisks indicate statistically significant differences (unpaired Student's t-test, $P < 0.05$).

sequence and of the WRCVLREGPAGGCAWFNRHRL phage, but had no effect on the brain localization of the CENWWDGVC phage (Fig. 3). Thus the first two peptides, which were obtained from different libraries in two independent experiments, seem to bind to the same target molecule, possibly because of the similarity of the C terminus of the long peptide with the SRL motif. Differences in binding parameters may explain the greater susceptibility of the long motif to inhibition by the CLSSRLDAC peptide. The third peptide is likely to have a different target.

We also showed that CLSSRLDAC could target a particle other than the phage to the brain. Coupling the peptide onto the surface of red blood cells resulted in their accumulation in the brain to a greater extent than in the kidney (Fig. 4). Moreover, the brain localization of the red blood cells was blocked by co-injection of the soluble peptide, whereas the accumulation in the kidney was not affected (Fig. 4).

Future studies will be needed to identify the molecules to which the peptides bind in the brain and kidney. The sequences of the binding motifs are not helpful in this regard, because they do not reveal any significant similarities with known receptor ligands. Our initial attempts to identify the target molecule for the CLSSRLDAC peptide by affinity chromatography of brain extracts have not been successful, possibly because endothelial cell molecules would only be present as minor components in a brain extract. The receptors for the peptides are likely to be endothelial cell molecules, because the phage were allowed to circulate only for a few minutes, making it unlikely that the phage would have left the circulation. Moreover, immunohistochemical staining of phage after injection showed that they remain in the lumen of blood vessels in the targeted organs.

To our knowledge this is the first time an *in vivo* selection procedure has been applied to a random library. So far we have targeted only two organs, the brain and the kidney, and were in each case able to recover organ-selective phage. This initial success suggests that it will be possible to apply this procedure to the identification of selective binding sequences for other organs as well, although organs that capture a large number of phage, such as liver and lung, may prove rather more troublesome. The method should be applicable to phage display libraries expressing larger proteins including the antibody variable binding region and the binding domains of specific ligands, as well as random libraries based on principles other than phage display; the only requirement is the ability to identify the compound in the tissue after the *in vivo* binding.

Organ-selective targeting molecules isolated from random libraries following the procedures described here may have a variety of uses. It may be possible to graft motifs to surface molecules of viruses or cells used in gene therapy. Other possibilities include their use in the preparation of drug conjugates or liposomes with specific targeting properties. Tumour vasculature, which undergoes active angiogenesis and contains specific markers[14,15], would be a particularly attractive future target, as it might allow therapies to be directed into tumours while sparing other tissues. □

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CORRESPONDENCE AND MATERIALS. Requests to be addressed to E.R. (e-mail address ruoslahti@ljcrf.edu).

CORRECTION

Behavioural and cardiovascular effects of disrupting the angiotensin II type-2 receptor gene in mice

Lutz Hein, Gregory S. Barsh, Richard E. Pratt, Victor J. Dzau & Brian K. Kobilka

Nature 377, 744–747 (1995)

REFERENCES 22 and 25 of this paper should be replaced as follows:

22. Nakajima, M. *et al. Proc. natn. Acad. Sci. U.S.A.* **92,** 10663–10667 (1995).
25. Viswanathan, M. & Saavedra, J. M. *Peptides* **13,** 783–786 (1992). □

Molecular Heterogeneity of the Vascular Endothelium Revealed by In Vivo Phage Display

Daniel Rajotte,* Wadih Arap,* Martin Hagedorn,* Erkki Koivunen,‡ Renata Pasqualini,* and Erkki Ruoslahti*

**Research Center, The Burnham Institute, 10901 North Torrey Pines Road, La Jolla, California 92037; and ‡Department of Biochemistry, Biocenter 1B, Viikinkaari 5, PL56, FIN-00014, University of Helsinki, Helsinki, Finland*

Abstract

Vascular beds are known to differ in structure and metabolic function, but less is known about their molecular diversity. We have studied organ-specific molecular differences of the endothelium in various tissues by using in vivo screening of peptide libraries expressed on the surface of a bacteriophage. We report here that targeting of a large number of tissues with this method yielded, in each case, phage that homed selectively to the targeted organ. Different peptide motifs were recovered from each of these tissues. The enrichment in homing to the target organs relative to an unselected phage was 3–35-fold. Peptide sequences that conferred selective phage homing to the vasculature of lung, skin, and pancreas were characterized in detail. Immunohistochemistry showed that the phage localized in the blood vessels of their target organ. When tested, the phage homing was blocked in the presence of the cognate peptide. By targeting several tissues and by showing that specific homing could be achieved in each case, we provide evidence that organ- and tissue-specific molecular heterogeneity of the vasculature is a general, perhaps even universal, phenomenon. Our results also show that these molecular differences can serve as molecular addresses. (*J. Clin. Invest.* 1998. 102:430–437.) Key words: blood vessels • vascular markers • endothelial cells • organ targeting • peptide libraries

Introduction

Vascular endothelial cells play a crucial role in many physiological processes, including blood–tissue exchange, activation and migration of white blood cells, fibrinolysis, and coagulation (1). The vascular system is important in a variety of pathological conditions, including the main causes of morbidity and mortality—atherosclerosis and cancer (2, 3).

There are indications that the vascular beds in different parts of the body are morphologically and functionally different (4, 5). This is particularly true of the lymphoid tissues, where the high endothelium is composed of cells that express unique adhesion molecules for lymphocyte homing (6–8). Moreover, metastasis into preferred organs by certain tumors may depend on interactions between tumor cells and organ-specific molecules in vascular beds. Thus, endothelial cell membrane vesicles have been found to bind preferentially those tumor cells that metastasize to the tissue of origin of the endothelial vesicles (9, 10).

Identification of organ-specific vascular markers has progressed slowly, at least partly because of difficulties in isolating pure populations of endothelial cells from tissues. Moreover, isolated and cultured cells may lose their tissue-specific traits upon culture (5, 11, 12). Thus, the phenotype of endothelial cells is unstable and likely to change when the cells are removed from their microenvironment.

Phage display peptide libraries are commonly used to obtain defined peptide sequences interacting with a particular molecule. In this system, peptides in as many as 10^9 permutations are expressed on the phage surface by fusion to one of the phage surface proteins and the desired peptides are selected on the basis of binding to the target molecule (13). The strength of this technology is its ability to identify interactive regions of proteins and other molecules without preexisting notions about the nature of the interaction. Phage libraries have been used to select for peptides that bind immobilized proteins (14–17), carbohydrates (18), and for peptides that bind to cultured cells (19).

Recently, we reported on a new approach to the use of phage libraries. We showed that peptides capable of tissue-specific homing could be identified by performing a selection for that trait in vivo (20). In that work, we isolated peptides capable of homing to two organs, the brain and the kidneys. The objective of the present study was to conduct a broad assessment of the organ-specific molecular diversity in endothelia. We have targeted seven different organs and obtained, in each case, phage that homed preferentially to the target organ. Detailed analysis of the homing to three of these organs revealed unique peptide sequences that mediated selective phage homing to the vasculature of these organs. As these tissues were selected to be different in size, location, function, and embryological derivation, our results suggest that a widespread system of endothelial specificities exists in tissues.

Methods

Phage peptide libraries. Fuse5 vector and K91kan bacterial strain were a gift from G. Smith (21). Peptide phage libraries were constructed as described previously (16). The libraries used in this study were: X_7, CX_6C, CX_7C, $CX_3CX_3CX_3C$, and X_2CX_4CX (C = cysteine; X = any amino acid). The titer of the various libraries was $\sim 10^{12}$ transducing units/ml (TU/ml).[1] Purification of phage particles and se-

Address correspondence to Erkki Ruoslahti, The Burnham Institute, 10901 N. Torrey Pines Road, La Jolla, CA 92037. Phone: 619-646-3125; FAX: 619-646-3198; E-mail: ruoslahti@burnham-inst.org

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1. *Abbreviations used in this paper:* GST, glutathione-S-transferase; TU, transducing units.

quencing of phage single-stranded DNA were performed as described (16).

In vivo selection. 2-mo-old Balb/c and Balb/c nude mice were purchased from Harlan Sprague Dawley (San Diego, CA). In vivo phage selection was performed as described previously, with a few modifications (20, 22). Briefly, mice were first anesthetized with 0.017 ml/g of Avertin and then injected intravenously (tail vein) with 10^{10} TU of phage peptide library. A major modification to the earlier procedure was that after 5 min of phage circulation in vivo, the mice were perfused through the heart with 5–10 ml of DME (Sigma Chemical Co., St. Louis, MO). Organs were then weighed, homogenized, and the phage were rescued by infection with K91kan bacteria. For the second and third rounds of in vivo selection, at least 200 clones were picked from the previous round and grown individually. Cultures were then pooled, phage particles purified, and 10^{9} TU of this pool were injected into mice. Phage ssDNA of individual clones from the second and third round of selection was prepared and the insert sequenced. Phage with sequences appearing repeatedly in both rounds were retained, and the selected phage were then characterized further by injecting them individually.

For in vivo targeting of the skin we used Balb/c nude mice to avoid contamination of the tissue with hair. The mice were injected intravenously with phage and perfused as described above. Any contact of the skin with perfused blood was avoided. Skin was removed in large sections and placed on an ice-cold plate with the hypodermis facing up. The skin was then scraped with a scalpel, yielding primarily the hypodermis, and leaving behind the epidermis and part of the dermis. The scraped skin tissue was then processed for phage recovery as described above.

For in vivo targeting of the retina, 2-mo-old female Simonson Albino rats were anesthetized using 50 mg/kg body weight sodium-phenobarbital. While under deep anesthesia, the rats were injected with 10^{10} TU of a library. The injection was administered into the left ventricle of the heart. After 2–5 min circulation time, the anterior chamber with cornea and lens were removed and the retina was peeled off the remaining posterior chamber. The tissue was homogenized in 1 ml ice-cold DME containing 1 mM PMSF, 20 μg/ml aprotinin, and 1 μg/ml leupeptin (all from Sigma Chemical Co.). The tissue was washed three times with 1 ml DME and containing protease inhibitors. The phage were rescued by infecting bacteria and used for the subsequent round of selection. To assess homing of individual phage to retina, a phage that provides ampicillin-resistance, fdAMPLAY88 (23; a gift from Richard N. Perham, Cambridge University, Cambridge, UK), was used as an internal control. Rats were injected with an equal amount of the selected and fdAMPLAY88 phage, and homing to retina was evaluated by comparing the number of TU rescued from the retina on tetracycline (selected phage) and ampicillin plates (fdAMPLAY88).

Glutathione-S-transferase (GST)–fusion proteins. PCR amplification of the peptide-coding inserts was performed on phage ssDNA using the primers: AGGCTCGAGGATCCTCGGCCGACGGGGCT (sense) and AGGTCTAGAATTCGCCCCAGCGGCCCC (antisense). Annealing temperature for PCR was 53°C. PCR products were ethanol precipitated and digested with BamHI and EcoRI. Digested fragments were inserted in-frame into the same sites of pGEX2TK vector (Pharmacia; Uppsala, Sweden). Large-scale preparations of GST-fusion proteins were produced and purified according to manufacturer's instructions (Pharmacia). The molecular weight and purity of the GST-fusion proteins were examined by SDS-PAGE.

Immunohistochemistry. Phage proteins were detected in tissues by immunostaining as described (20, 22). Briefly, anesthetized mice were injected with 10^{9} TU of phage and perfused as described above. The organs were surgically removed and then fixed in Bouin's solution (Sigma Chemical Co.). An antiserum against M13 phage (Pharmacia) was used for the staining, followed by a peroxidase-conjugated secondary antibody (Sigma Chemical Co.).

Results

Organ-selective phage isolated by in vivo screening. To identify phage that home selectively to individual organs, we injected phage libraries intravenously into mice and subsequently rescued the phage from these targets. We performed in vivo screens on lung, pancreas, skin, intestine, uterus, adrenal gland, and retina. All the screens yielded enrichment in phage homing to the target organ. The phage homing to lung, pancreas, and skin were selected for a detailed analysis.

Fig. 1 shows the enrichment profile obtained in three rounds of selection when targeting the lungs with a CX_6C li-

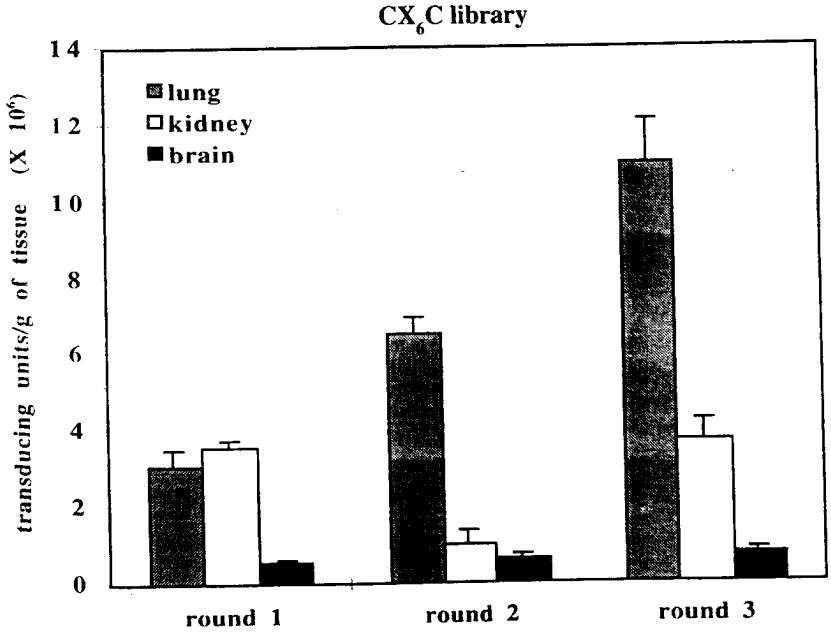

Figure 1. Selection of lung-targeting phage by in vivo screening of a phage-displayed peptide library. A CX_6C library (10^{10} TU) was injected into the tail vein of mice. 5 min after the injection, the mice were perfused through the heart and phage were rescued from various organs. Phage recovered from the lungs was amplified and re-injected in two consecutive rounds. The number of TU recovered from lung, kidney, and brain tissue is shown. Error bars show standard deviation of the mean from triplicate platings.

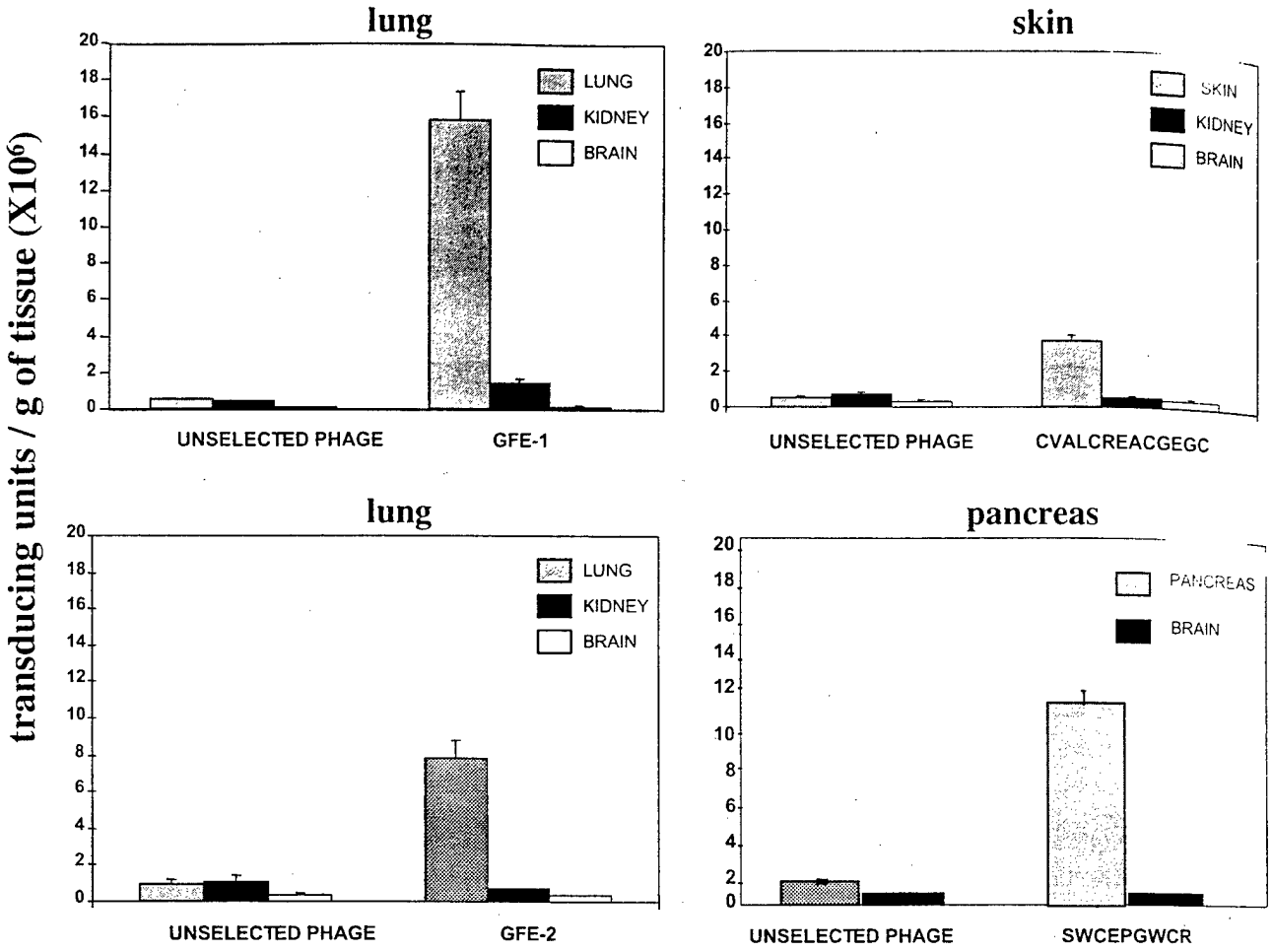

Figure 2. Specificity of organ-homing phage. Mice were injected with 10^9 TU of phage selected for organ homing or with unselected phage without insert. 5 min after the injection, the mice were perfused and phage were recovered from the indicated organs. The number of TU recovered is shown. Selective phage homing is shown for lung (GFE-1 and GFE-2), skin (CVALCREACGEGC), and pancreas (SWCEPGWCR) phage. Error bars show standard deviation of the mean from triplicate platings.

brary. The number of TU recovered from lung tissue markedly increased in the second and third rounds of selection, whereas the number of TU recovered from the kidney and the brain, which were used as controls, remained largely unaffected. In an earlier study, we observed an increase in phage homing to kidney and brain when targeting specifically these organs (20). The enrichment in the number of phage homing to lung upon selection was consistent and was also observed with libraries that had the general structures $CX_3CX_3CX_3C$ and CX_7C (data not shown).

Phage that displayed the same or related peptide sequence appeared repeatedly in both second and third rounds of the lung screening. The lung-homing potential of these selected phage was then characterized further by injecting them individually. Four individual phage that homed to the lungs were identified. Two of these phage, isolated in independent screens, share the tripeptide GFE. The CGFECVRQCPERC (termed GFE-1) phage showed 35-fold and the CGFELETC (termed GFE-2) phage ninefold enrichment in homing to lung relative to an unselected phage (Fig. 2; Table I). No specific homing to kidney or brain could be detected. Two additional lung-homing phage from the CX_6C library gave a sixfold

Table I. Targeting Sequences

Organ/tissue	Displayed sequence	Homing (Fold over unselected phage)
—	None	1
Lung	CGFECVRQCPERC (GFE-1)	35
Lung	CGFELETC (GFE-2)	9
Lung	CTLRDRNC	6
Lung	CIGEVEVC	5
Skin	CVALCREACGEGC	7
Pancreas	SWCEPGWCR	6
Intestine	YSGKWGW	[illegible]
Uterus	GLSGGRS	3
Adrenal gland	LMLPRAD	4
Retina	CSCFRDVCC	3*
Retina	CRDVVSVIC	2*

The relative level of organ homing for each phage, compared to a phage without insert, is shown. Underlined sequences indicate common motifs displayed from either lung (*double underlined*) or retina (*single underlined*). *For retina, the level of phage homing was determined by using fdAMPLAY88 ampicilin-resistant phage as an internal control.

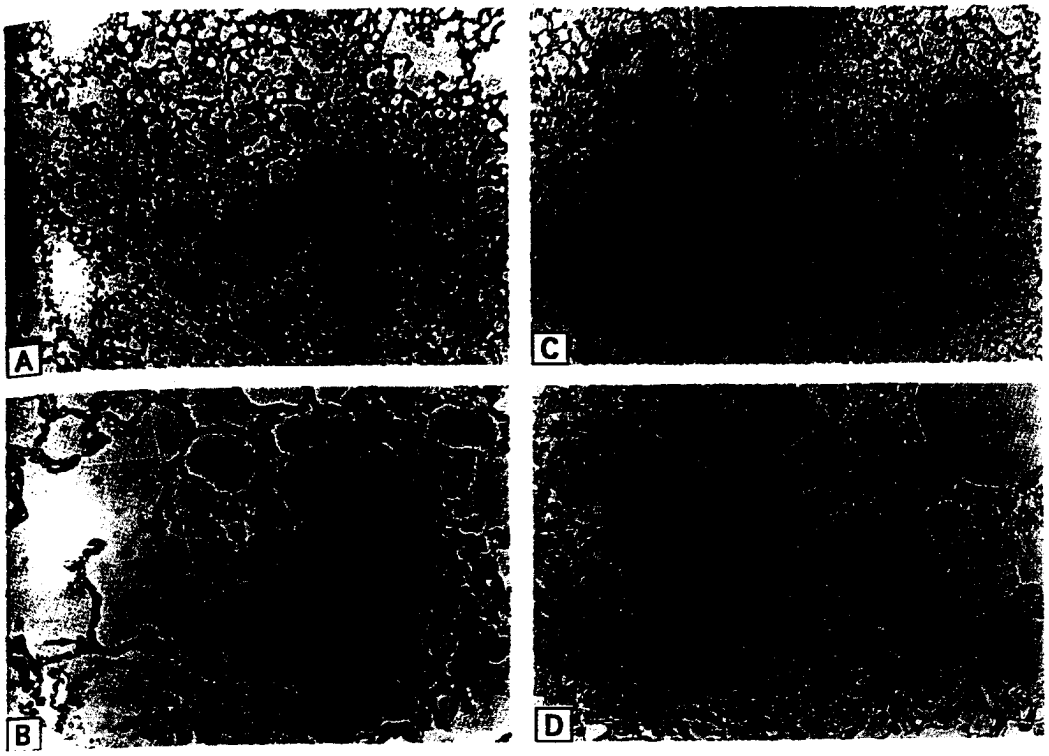

Figure 3. Immunohistochemical detection of phage homing to lung vasculature. GFE-1 (*A* and *B*) or unselected (*C* and *D*) phage were injected (10^9 TU) into mice and allowed to circulate in vivo for 5 min. After perfusion, tissue sections were prepared and subjected to immunoperoxidase staining to detect phage proteins. In *B*, small arrows are pointing to representative capillaries positive for phage staining. As indicated by the large arrow, larger blood vessels do not bind GFE-1 phage. Magnification: *A* and *C*: 100×; *B* and *D*: 400×.

(CTLRDRNC phage) and fivefold (CIGEVEVC phage) enrichment over unselected phage (Table I). Interestingly, the EVE sequence in CIGEVEVC resembles the ELE tripeptide in GFE-2.

Panning of skin with a $CX_3CX_3CX_3C$ library also yielded enriched phage homing (data not shown). The peptide sequence CVALCREACGEGC appeared repeatedly in the second and third rounds of the selection. When tested individually, this phage gave sevenfold selectivity for skin over unselected phage and over the background in brain and kidney (Fig. 2; Table I).

In a screen for the pancreas, the sequence SWCEPGWCR appeared multiple times in the second and third rounds of selection. Phage displaying this sequence showed approximately sixfold enrichment in homing to pancreas compared to unselected phage and the background in the brain was low (Fig. 2; Table I).

In vivo screens for intestine, uterus, adrenal gland, and retina yielded phage showing selective homing to these tissues. The peptide displayed by these phage and the corresponding enrichment are summarized in Table I.

Immunohistochemical staining for homing phage reveals vascular localization and organ specificity. Strong immunoperoxidase staining for phage was seen in lung vasculature 5 min after intravenous injection of the GFE-1 lung-homing phage (Fig. 3, *A* and *B*). Alveolar capillaries were stained, whereas bronchiolar walls and some larger blood vessels were negative. No preference for any anatomical part of the lung was seen. Mice injected with unselected phage did not show staining in the lung (Fig. 3, *C* and *D*). The GFE-2 phage was also detected by staining in the lung microvasculature, but the signal was weaker than for GFE-1 (data not shown). This weaker staining intensity of GFE-2 agrees with the phage-counting data, which also showed more lung binding by GFE-1 than GFE-2 (Fig. 2).

The skin-homing phage was detected on blood vessels of the hypodermis 5 min after intravenous injection; the dermis was not consistently stained (Fig. 4, *A* and *B*). Injecting an equal amount of unselected phage caused no staining in the blood vessels of the hypodermis or dermis (Fig. 4, *C* and *D*). To determine whether the phage had access to the dermal blood vessels, we injected unselected phage at a 50-fold higher input than was used with the skin-homing phage and omitted the perfusion step. Although this procedure gave background staining in the deep dermal vessels, only occasional vessels of the dermis contained phage. This result suggests that circulating phage may not gain sufficient access to the dermal vessels to cause immunostaining. Thus, the apparent lack of dermal homing by the skin-homing phage may be due to poor access of the phage to these vessels rather than lack of a receptor.

The pancreas-homing SWCEPGWCR phage was found both in capillaries and larger blood vessels of the exocrine pancreas (Fig. 5 *A*) and in the pancreatic islets (Fig. 5 *B*). Again, an unselected phage caused no staining (Fig. 5, *C* and *D*).

The lung- and skin-homing phage caused no staining in any of the other organs tested. These organs include kidney, brain, heart, muscle, lymph nodes, pancreas, intestine, and uterus (data not shown). The SWCEPGWCR pancreas–homing phage was an exception; it did give some staining in blood vessels within the uterus. Moreover, we recovered sixfold more SWCEPGWCR phage than unselected phage from the uterus after an intravenous injection (data not shown). Thus, this phage homes both to the pancreatic and uterine vasculature. All phage, including the unselected phage, caused staining in

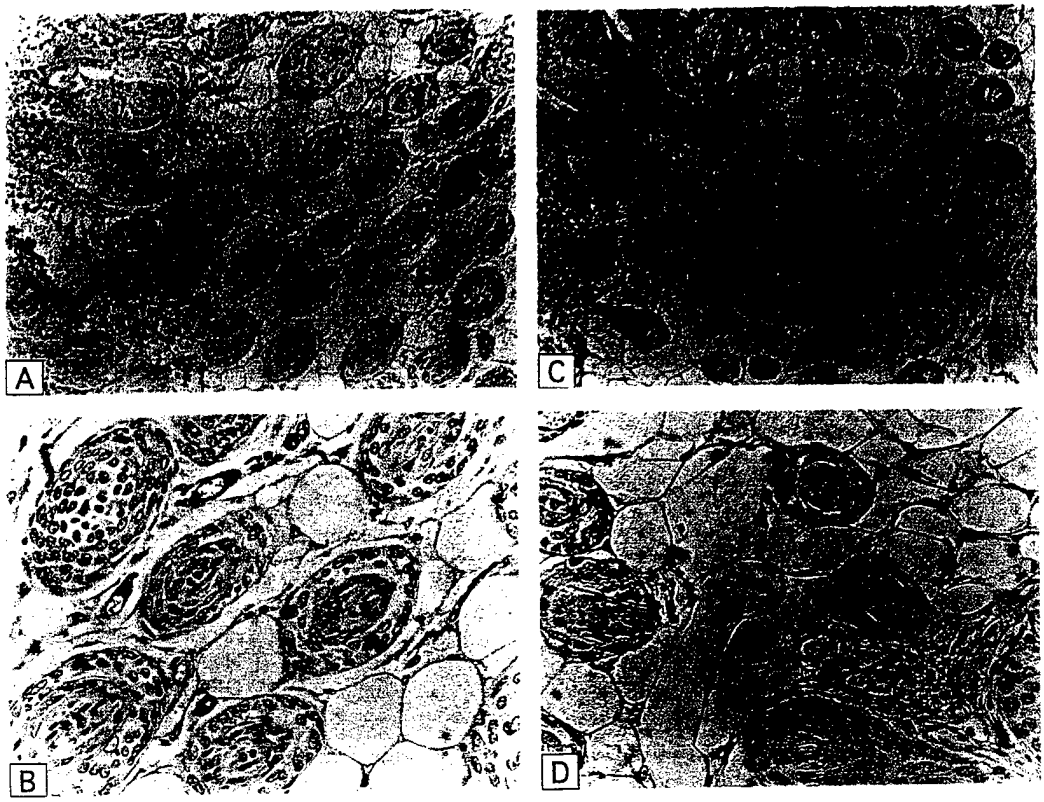

Figure 4. Immunohistochemical detection of phage homing to skin vasculature. Equal amounts of the CVALCREACGEGC phage (*A* and *B*), or unselected phage (*C* and *D*), were injected into the tail vein of individual mice and allowed to circulate for 5 min. After perfusion, sections were prepared and stained for the presence of phage proteins. Magnification: *A* and *C*: 100×; *B* and *D*: 400×.

the liver and spleen (data not shown). This is consistent with the previously described capture of circulating phage by the reticuloendothelial system (20, 22, 24).

Cognate peptides compete for phage binding in vivo. The enrichment of certain sequences strongly suggested that the phage homing to target tissues depended on the peptide displayed by the phage, and not on some incidental mutant property of the phage. To confirm the role of the phage-displayed

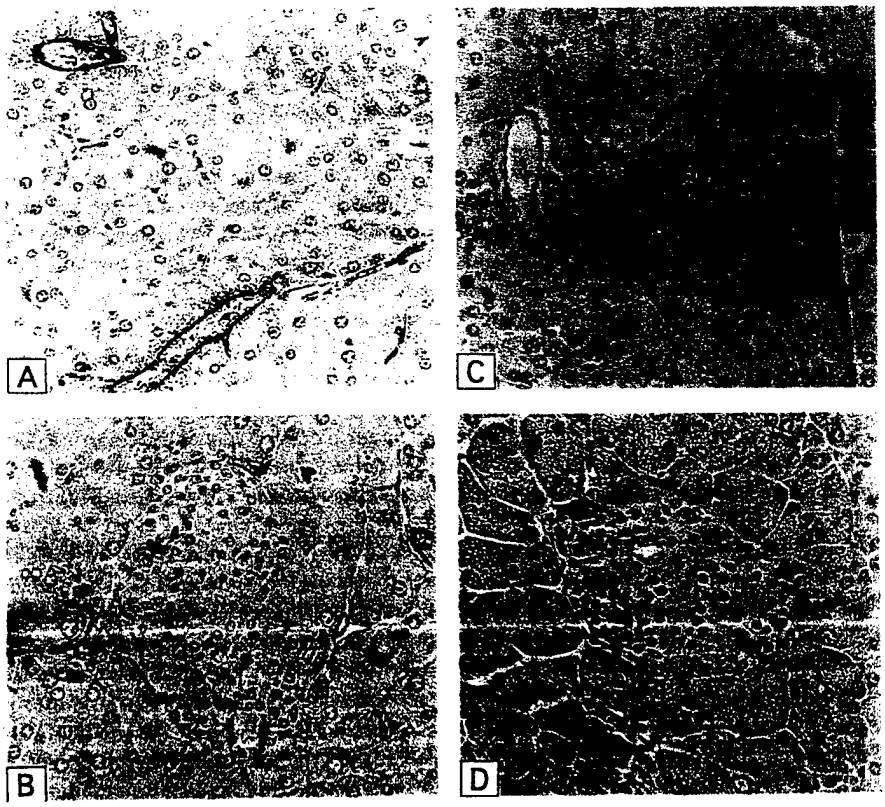

Figure 5. Immunohistochemical detection of phage homing to pancreas vasculature. Equal amounts of the CWCEPGWCR phage (*A* and *B*), or unselected phage (*C* and *D*), were injected into the tail vein of individual mice and allowed to circulate for 5 min. After perfusion, sections were prepared and stained for the presence of phage proteins. The exocrine pancreas (*A* and *C*) and islets (*B* and *D*) are shown. Magnification: 160×.

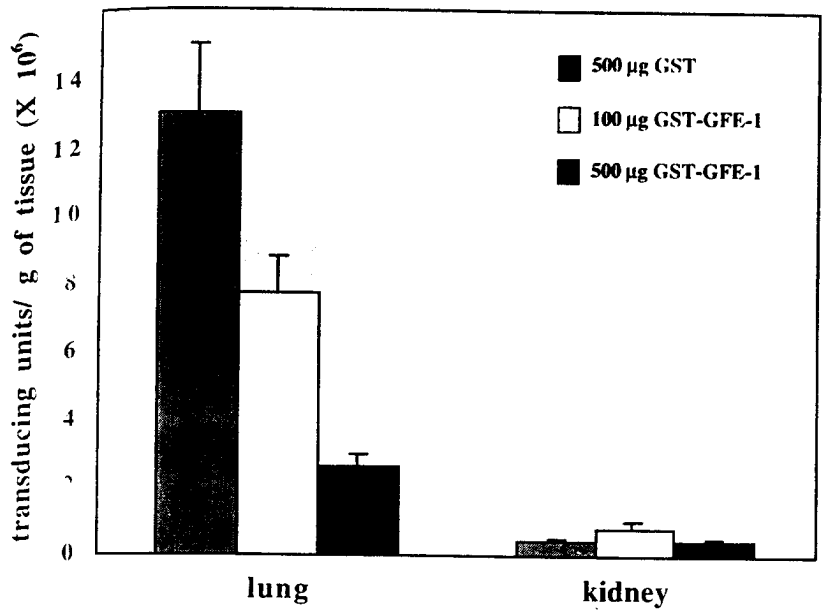

Figure 6. Effect of GST–GFE-1 fusion protein on GFE-1 phage homing in vivo. GST or GST–GFE-1, at the indicated amount, were injected into mice together with 10^9 TU of GFE-1 phage. 5 min after injection, phage were recovered from lung and kidney. Error bars indicate standard deviation of the mean from triplicate platings.

sequences in the targeting to specific organs, we constructed GST fusion proteins containing these sequences and used them as competitors for phage homing in vivo. A dose-dependent inhibition of the GFE-1 phage homing to lung tissue was observed when the GST–GFE-1 protein was co-injected with the phage; 500 μg of GST–GFE-1 inhibited phage homing by 70% (Fig. 6). Co-injection of 500 μg of GST protein did not affect lung homing of this phage. Moreover, GST–GFE-1 had no effect on the nonspecific recovery of phage from the kidney (Fig.). Lung homing of the GFE-2 phage was partially inhibited by the corresponding GST-fusion protein. At 500 μg, GST–GFE-2 inhibited the homing by 30% in several independent experiments. Higher concentrations did not improve the inhibition. The GST–GFE-1 protein at 500 μg inhibited lung homing of the GFE-2 phage by 60%, but the same amount of the GST–GFE-2 protein had no effect on the homing of the GFE-1 phage. Possible reasons for the lack of GST–GFE-2 effect on the GFE-1 phage homing include that GFE-1 may have a higher affinity for a shared receptor, or that the GFE-2 fusion protein may aggregate at higher concentrations.

GST–CVALCREACGEGC inhibited the homing of CVALCREACGEGC phage to skin by 55%. An equal amount of GST alone had no effect. The GST–CVALCREACGEGC protein also had no effect on the phage recovery to other organs such as lung and brain. There was no cross-inhibition among the lung-homing and skin-homing peptides: the GST–CVALCREACGEGC protein had no effect on the homing of the GFE phage; the GFE fusion proteins did not affect the CVALCREACGEGC phage (data not shown).

The homing of the SWCEPGWCR phage to pancreas was not inhibited reproducibly by the corresponding GST-fusion protein (data not shown). This lack of inhibitory activity could be due to a difference in the conformation of the peptide displayed by the two systems or to a higher binding affinity of the phage-displayed peptide than the GST-fusion protein for their ligand—multivalent display of a peptide can lead to an increase in binding affinity (16).

Discussion

We show here that vascular beds of a large number of tissues differ from one another, and we describe peptide sequences—identified by in vivo screening of phage-displayed peptide libraries—that are capable of homing selectively to the vasculature of the lung, the skin, and the pancreas. Together with earlier results (20), these findings establish a system of tissue specific individuality of vascular beds.

It may seem surprising that our method consistently yields tissue-specific homing peptides, rather than peptides that home nonselectively to any tissue. The phage libraries contain only few phage displaying of any given sequence. Injecting 10^{10} phage particles into a mouse means that < 100 copies of any given phage are included. As this is a small number, any phage that binds to a ubiquitous endothelial marker will interact with multiple binding sites elsewhere than in the tissue of interest and will be rapidly depleted from the circulation. Thus, we believe that the selectivity is based on elimination of nonspecific phage binding at nontargeted sites. This would leave only those phage capable of binding selectively to the intended target tissue to be enriched there.

The potential of in vivo phage display for the identification of targeting sequences is obviously not exhausted, because we have tested relatively few types of libraries. Moreover, there would seem to be a variety of organ-specific markers targetable by our method, because it often yielded peptides with unrelated sequences that can home to the same vascular beds. However, as illustrated by the GFE (lung) and the RDV (retina) motifs, we may have targeted the same molecules repeatedly. These results indicate that a given vascular bed may have multiple specific markers, but that the number of such markers is finite.

We have, in this study, improved on our homing peptide selection by perfusing the mice after the injected phage had circulated. This reduced the nonspecific background of phage recovery. This was particularly useful in the targeting of the

lungs, which we had previously found to yield high levels of background phage (20). In this study, we describe a protocol in which perfusion of the mice after phage circulation allows one to reduce that background. In addition, perfusion may increase the stringency of the screen, selecting for phage with higher affinities for the target. Moreover, we have recently developed an in vivo selection approach that includes noninfective phage as a competitor to thwart phage trapping in organs containing reticulo-endothelial tissue, such as liver and spleen. Thus, screening for peptides that home specifically to these tissues may also be possible (R. Pasqualini, E. Koivunen, and E. Ruoslahti, unpublished results).

It is interesting that the binding motif in the targeting peptides is often a tripeptide that appears in different sequence contexts. In in vivo screens for brain-homing phage the SRL tripeptide was found in several peptides (20). In the present work, we found a GFE motif in two lung-homing peptides and an RDV motif in two retina-homing peptides. In a different but related situation, the RGD motif is known to be important for integrin binding in distinct molecular contexts (25). Thus, many adhesive interactions seem to derive their specificity from tripeptide recognition motifs.

Our homing peptides bind to the vasculature in the target tissues. Earlier results have documented that for peptides homing to the brain, kidney, and several tumors (20, 22, 26). Thus, the vasculature of many tissues carries a tissue-specific signature in the form of markers that can be detected by our phage display–based method. In some organs, such as pancreas and uterus, this signature may be shared by unrelated tissues. We have also obtained peptides that target the prostate with high selectivity (W. Arap, R. Pasqualini, and E. Ruoslahti unpublished results). Thus, including the brain and kidney homing reported earlier (20), the prostate and the seven organs reported here, the total number of organs targeted so far is ten. Given that we have been successful with every organ we have chosen to target, one can speculate that all tissues may "label" their vasculature with distinct markers.

There are other reports on tissue-selective molecular differences in endothelia (27). Possibly related to our findings on specific phage homing to the lung, dipeptidyl peptidase has been found to be expressed selectively in the lung endothelium and to serve as the receptor for a lung-metastasizing tumor cell line (28). Recently, Ghitescu et al. developed antibodies specific to the plasma membrane of rat lung microvascular endothelium (29). Two of the proteins detected by their antibodies show similar tissue distributions to our GFE phage. Direct comparison of the target molecules for the antibodies and our peptides is underway to resolve the question regarding their identity or dissimilarity. Moreover, surface protein differences have been detected by *Dolichos biflorus* agglutinin binding (30) and in situ glycoprotein labeling in endothelial surface proteins from liver, kidney, and brain (31). Finally, Aird et al. (32) recently showed that expression of the von Willebrand factor by endothelial cells can be regulated by organ-specific transcriptional pathways. These observations are in agreement with our results. However, the extent of tissue-specific molecular individuality of various vascular beds revealed by our data is unprecedented.

Our data also reveal molecular heterogeneity of the endothelium within a given organ. In the lungs, the GFE-1 and GFE-2 phage stained mainly capillaries rather than larger blood vessels. Interestingly, in the embryonic lung, capillaries do not arise as extensions of major blood vessels growing from the heart. Rather, the angioblasts within the lung give rise to capillaries that eventually become linked to extensions of the major blood vessels (33). Thus, our results may begin to delineate the molecular correlatives of such changes.

Our group has recently used in vivo selection of phage display libraries to isolate peptides that home specifically to tumor blood vessels (26). When coupled to the anticancer drug doxorubicin, these peptides enhanced the efficacy of the drug against human breast cancer xenografts in nude mice and also reduced its toxicity. Thus, our tissue-specific homing peptides may not only contribute to a better understanding of endothelial biology, but can also provide molecular tools for targeting individual vascular beds with diagnostic probes and therapeutic substances.

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RESULTS OF BACTERIOPHAGE TREATMENT OF SUPPURATIVE BACTERIAL INFECTIONS

I. General evaluation of the results

by

STEFAN ŚLOPEK, IRENA DURLAKOWA, BEATA WEBER-DĄBROWSKA, ALINA KUCHAREWICZ-KRUKOWSKA, MAREK DĄBROWSKI and REGINA BISIKIEWICZ

Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Czerska 12, 53-114 Wrocław

One hundred and thirty eight septic cases were treated with specific bacteriophages. According to the International Classification of the Diseases (WHO, 1977), the treated cases were divided to 9 categories. Nearly all cases were long-term infections with antibiotic resistant organisms. Only specific bacteriophages were used in association with several types of surgical procedure. The technique of treatment is described. In 129 (93.5%) cases the results were good, in 9 (6.5%) cases local improvement was observed. It is concluded that bacteriophage therapy may be helpful in the treatment of long-term suppurative infections.

The idea of bacteriophages (phages) application in the treatment of infectious diseases of bacterial origin is not new. Bacteriophage (phage) therapy was initiated in 1921 by BRUYNOGHE and MAISIN [3] in the treatment of staphylococcal skin diseases.

However, this new form of therapy of suppurative bacterial infections did not evoke any special interest during 20 interwar years.

The degree of interest with phage therapy showed substantial fall together with the introduction of sulphonamides and then antibiotics. Renewed interest in this problem emerged again 40 years ago and was connected with the appearance of more and more frequent and difficult for curing infections induced by antibiotic and chemotherapeutic-resistant bacteria [2, 4]. This concerned mainly suppurative postoperative and hospital infections induced not only by antibiotic-resistant *Staphylococci* but also by Gram-negative bacteria as: *Pseudomonas, Klebsiella, Escherichia Proteus* and, rarely, by other types of bacteria.

In spite of great interest in phage therapy, majority of reports encountered in the literature are of casuistic type and those based on vast clinical material evaluating the curative value of phage therapy, are rather scarce.

The present work consists of two parts. The first one analyzes the results of application of bacteriophages in 138 cases; the second, presents a detailed analysis of phage therapy in 184 categories of various diseases diagnosed clinically in 138 patients treated with bacteriophages.

MATERIALS AND METHODS

Characteristics of the investigated groups of patients

Phage therapy was applied in 138 patients with septic infections resulting from either idiopathic infections or post-operative complications. Age and sex of patients subjected to phage therapy is presented in Table 1. The age 21–77 years was predominating (78% patients). The ratio of men to women was almost equal, the number of men being slightly predominating (55.8%:44.2%). The classification into categories was based on the recommendations of the World Health Organization presented in the publication: "Manual of the International Statistical Classification of Diseases, Injuries and Causes of Death", vol. 1, WHO, Genewa 1977[6]. The number of nosologic units in particular categories is depicted in Table 2.

Table 1. Age and sex of patients treated with bacteriophages

Age of patients	No. of cases	Sex	
		M	F
Up to 1 year of age	6 (4.4%)	2	4
1–5	11 (8.0%)	5	6
6–10	5 (3.6%)	1	4
11–20	18 (13.0%)	11	7
21–30	30 (21.7%)	19	11
31–40	15 (10.9%)	10	5
41–50	23 (16.7%)	14	9
51–60	14 (10.1%)	6	8
61–70	15 (10.9%)	9	6
Above 70 years of age	1 (0.7%)	0	1
Together	138	77 (55.8%)	61 (44.2%)

Phage therapy was carried out in 10 clinical and hospital departments, mainly on surgical departments (Institute of Surgery, Medical Academy, Wrocław: Cardiosurgery Clinic, Children Surgery Clinic, Orthopaedic Clinic; Institute of Internal Diseases: Nephrologic Clinic, Clinic of Pulmonary Diseases; K. Dłuski Lung Disease Hospital, Wrocław: Department of Immunotherapy; T. Marciniak Hospital in Wrocław: Department of Children Surgery; Hospital and Polyclinic of KWMO in Wrocław: Traumato-Orthopaedic Department; Department of Traumato-Orthopaedic Surgery of ZOZ in Lubin; Department of ZOZ Surgery in Oleśnica; Provincial Hospital of Locomotory Organs Diseases in Kamienna Góra).

Table 2. Disease categories according to the International Classification of Diseases (WHO, 1977)

Disease category	Number of cases
I. Infectious diseases	2 1.4%
III. Immunological disorders (humoral, cellular)	2 1.4%
VII. Diseases of the circulatory system (varicose ulcers of lower extremities)	8 5.8%
VIII. Disease of the respiratory system (mucopurulent inflammation of the upper respiratory tract, pneumonia, bronchopneumonia, suppurative pericarditis, mediastinitis)	17 12.3%
IX. Diseases of the digestive system (diseases of the oral cavity, suppurative peritonitis)	17 12.3%
X. Diseases of the genitourinary system (glomerulonephritis, urinary tract infections)	6 4.4%
XII. Diseases of the skin and subcutaneous tissue (furunculosis, abscesses cutis, decubitus ulcer)	20 14.5%
XIII. Diseases of the musculoskeletal system and connective tissue (pyogenic arthritis, infective myositis, postoperative ostitis)	31 22.5%
XVII. Injuries (contusions, postoperative wounds, burns, open wounds, pyogenic infections after fracture of bones)	35 25.4%
Total	138

Characteristics of infections in the patients examined

Out of 138 cases investigated, in 90 monoinfections and in 48 — polyinfections were confirmed Most frequent cause of monoinfection (Table 3) were pyogenic *Staphylococci* (67.7%), rarely *Pseudomonas* (14.4%), *Escherichia* (10.0%) and *Klebsiella* (10.0%) bacilli.

In the cases of polyinfection (Table 4), beside *Pseudomonas aeruginosa* (60.4%), most commonly (solated were pyogenic Staphylococci (70.8%). Rarely occurring were: *Escherichia* (33.3%), *Klebsiella* i 31.2 %), *Proteus* (20.2%) bacilli. The remaining microorganisms were isolated sporadically.

Table 3. Species of bacteria in monoinfections

Species of bacteria	No. of cases (n = 90)
Salmonella enteriditis	1 1.1%
Shigella sonnei	1 1.1%
Staphylococcus pyogenes	61 67.8%
Klebsiella pneumoniae	5 5.6%
Escherichia coli	9 10.0%
Proteus vulgaris	0
Pseudomonas aeruginosa	13 14.4%

Table 4. Species of bacteria in polyinfections

Species of bacteria	Number of cases (n = 48)
Staphylococcus pyogenes	34 70.8%
Klebiella pneumoniae	15 31.2%
Escherichia coli	16 33.3%
Proteus vulgaris	14 29.2%
Pseudomonas aeruginosa	29 60.4%
Streptococcus fecalis	2 4.2%
Enterobacter sp.	2 4.2%

Characteristics of bacteriophages applied

*Origin of bacteriophages**. In the experiments the use was made of 259 virulent bacteriophages including 116 for *Staphylococcus* of human and animal origin, 42 for *Klebsiella*, 39 for *Escherichia*, 30 for *Shigella*, 20 for *Pseudomonas*, 11 for *Proteus* and 1 for *Salmonella enteritidis*. In the course of therapy 52 *Staphylococcus* bacteriophages were used, 20 for *Escherichia*, 17 for *Klebsiella*, 17 for *Pseudomonas*, 4 for *Proteus* and for *Shigella* and *Salmonella* — one for each.

During therapy the use was made of the lysates of virulent bacteriophages which caused a total lysis of the sensitive bacterial strains isolated from the patients.

Isolation of bacterial strains from patients and determination of their sensitivity to bacteriophages. The bacteria from 18 h broth culture were passaged in broth and after 4 h incubation at 37°C (shaker), 2—3 ml of the suspension was sneared on a dried plate with WAHL et al. [7] medium (for *Staphylococci*) and on agar plates with phosphate buffer (for Gram-negative bacteria); an excess of the suspension was removed. One drop of bacteriophage (corresponding to the type of strain isolated) was applied to each plate after drying (30 min). For typing, the phages were diluted 1:10. The plates were incubated at 37°C for 4 h in the case of *Staphylococcus*, for 2—3 h with *Escherichia*, *Shigella* and *Klebsiella* and for 4—5 h with *Pseudomonas*. Thereafter, the plates were transferred into refrigerator and the result was read on a following day.

Propagation of Staphylococcus bacteriophages. After selection, the phages bringing about a total lysis on the strain isolated from patient, were propagated in 0.5 ml flasks containing 300 ml of broth, 5 ml of 30% glucose and 2.5 ml of 18 h *Staphylococcus* broth culture. After 1 h incubation at 37°C either 5 ml of phage lysate or agar from the spot showing full lysis, were added and the mixture was reincubated on a shaker for about 3 h. The strain culture on the same medium but without phage addition constituted the control. After lysis the flasks were stored overnight in a refrigerator. On the following day, the lysate was tested for the phage presence. If the lysate was turbid, it was centrifuged for 1 h at 3,500 rpm. After confirmation of the phage presence, the lysate was titrated and thymol crystal was added to kill the remaining bacterial cells. After 3-day storage in a refrigerator, the lysate from above thymol was collected and poured into ampouls, 10 ml to each and tested for sterility.

Propagation of bacteriophages for Gram-negative bacilli (Pseudomonas, Klebsiella, Escherichia, Proteus, Shigella and Salmonella). Pseudomonas phages were propagated in 10 ml of peptone water to which a young (4h) bacterial culture in amount 1—2 ml and 5 ml of phage lysate, was added. The control consisted of a flask with the same medium but without the phage. After completion of the lysis the flasks were transferred into refrigerator. On the following day, the lysate was tested for the presence of phage. To kill the remaining bacteria, 2 ml of chloroform was added to the lysates,

* The bacteriophages came from the collection of the Bacteriophage Laboratory of the Institute of Immunology and Experimental Therapy Polish Academy of Sciences, Wrocław.

shaken for 2 min, left for 2 h at room temperature, transferred into refrigerator and on the next day, the lysate from above chloroform was collected, poured into ampoules and tested for sterility.

Propagation of the remaining Gram-negative bacilli was carried out similarly except that the bacteria were cultured on broth instead of peptone water. Incubation lasted for 2 h and the propagations process of phages — about 3 h.

Therapeutic use of bacteriophages. Bacteriophages were administered orally 3 times a day, in amount of one 10 ml ampoule, 30 min before meal, after neutralization of the gastric juice (gelatum, baking soda or a glass of Vichy water). When applied directly on a wound, moist applications were recommended 3 times per twenty four hours.

When the phage was applied locally, it was made sure that the wound was not washed with any antiseptics to prevent inactivation of phages. If necessary, aseptic broth or physiological salt solution was used.

The bacteriophages were stored at $+2 - +4$°C. Intravenous administration of phages was not recommended for fear of possible shocks.

Clinical evaluation of the treatment

In the course of treatment regular bacteriological control and evaluation of sensitivity of the isolated bacteria to the bacteriophages aplied were carried out. If necessary, the bacteriophage was changed. In the case of a negative culture, the phages were continued to be applied prophylactically for 14 days.

Final evaluation of phage therapy was based on the notes from case history and on the data from a special inquiry. The inquiry was filled by a therapeutist and sent to the Institute of Immunology and Experimental Therapy, Polish Academy of Sciences in Wrocław. The results of the treatment were evaluated by a therapeutist.

The results of phage therapy were evaluated according to four-degree scale: ++++ outstanding effect manifesting by a complete recovery, +++ marked effect manifesting by a complete healing of the local lesions and liquidation of the suppurative process, ++ marked improvement of the local state with a tendency to healing of the lesions and negative result of bacteriological culture, + transitory improvement of the local state. In the last group the evaluation met with some difficulties.

These cases "difficult for evaluation" are separately discussed.

Elaboration of the results obtained

The results obtained were used for the elaboration of a computer program. This program was written in ALGOL 1900 language for Odra 1300 computer series and set to work at the Computation Center at Wrocław Technical University. The source program together with its numerical and technical characteristics is at disposal at the Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Wrocław.

Statistical evaluation of results was based on the following tests:

1. Chi-square goodness of fit test[5]:

$$\chi^2 = \sum_{i=1}^{k} \frac{n_i - np_i}{np_i}.$$

Number of degrees of freedom $r = k-1$.

2. *t*-test of the differences between means from two independent samples [1], assuming that $\sigma_1^2 = \sigma_2^2$:

$$t = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{s^2\left(\frac{1}{n_1} + \frac{1}{n_2}\right)}}$$

Number of degrees of freedom $r = n_1 + n_2 - 2$.

3. Test of inference on the basis of frequencies [4]:

$$u = \frac{|r - n\pi_o| - 1/2}{\sqrt{n\pi_o(1-\pi_o)}}.$$

4. Test of comparison of two frequencies of independent samples [4].

$$u = \frac{P_1 - p_2}{\sqrt{pq\left(\frac{1}{n_1} + \frac{1}{n_2}\right)}}.$$

5. Chi-square test of independence between two variables [5]:

$$\chi^2 = \frac{(E_i - O_i)^2}{O_i} \quad \text{or} \quad \chi^2 = \frac{(|E_i - O_i| - 1/2)^2}{O_i}$$

with Yate's correction.

Number of degrees of freedom:

$r = (k-1)\,(w-1)$ k — number of columns,

w — number of rows.

6. chi-square test of independence between two variables [5] taking into account the frequencies for the table "$w.2$"

$$\chi^2 = \sum \frac{x_i^2}{n_i} - \frac{(\sum x_i)^2}{\sum n_i},$$

w — number of rows,

Number of degrees of freedom: r = w − 1

7. Wilcoxon two-sample test for ranked observations[8]

$$W = \sum_{i=1}^{n} r_i.$$

RESULTS

Noteworthy is that in 125 cases (90.6%) out of 138 treated, phage therapy was performed on patients resistant to antibiotics and chemotherapeutics as the only way of inhibition and control of the infection. Only in 13 patients (9.4%) not treated previously with antibiotics, the bacteriophages were used.

Of 138 cases treated with bacteriophage , in 129 (93.5%) a good therapeutic result was obtained manifesting by a control of the infection and healing of the local lesions (Table 5).

Table 5. General evalu ation of phage therapy

No. of cases	Result of treatment			
	+	++	+++	++++
138	9	8	99	22
Percentage	6.5%	5.7%	71.7%	16.1%

Statistical evaluation of the results obtained during phage therapy was based on the chi-square test of goodness of fit (test 1) and on the test of inference on the basis of frequency (test 3). The results evidence that the effect of phage therapy in 138 subjects was not accidental, and point to great efficacy of the treatment (test 1) $\chi^2 = 164.3$, $p < 0.001$ with $r = 3$). As observed, in majority of the patients treated with bacteriophages, a complete recovery or a healing of the local lesions was obtained (test 3, $u = 8.77$, $p < 0.001$).

Results of phage therapy and the age of patients

Results of the treatment in relation to patients' age are depicted in Tables 6, 7 and 8. Table 6 presents the results of phage therapy, mean age of the patients and standard deviations. Statistical evaluation of the results obtained was based on the the t test of the differences between two means from independent samples, assuming that $\sigma_1^2 = \sigma_2^2$. Number of degrees of freedom — n_1+n_2-2 (test 2).

As follows from Table 6, the effect of phage therapy is related to the age of patients subjected to it. For instance, the difference of the average age of patients with

Table 6. Age of patients and result of phago therapy

	Result of treatment			
	+	++	+++	++++
No. of patients	9	8	99	22
(n = 138)	6.5%	5.7%	71.7%	16.1%
Average age (AA)	45.7	36.9	32.1	32.6
Standard deviation of age (SD)	16.2	21.1	20.2	20.1

Table 7. Age of patients divided into 4 categories and result of phage therapy

Result of treatment	0-	20-	40-	60-	No. of cases
+	1	2	4	2	9
++	1	4	1	2	8
+++	30	31	27	11	99
++++	6	7	6	3	22
No. of cases	38	44	38	18	138

Calculation of estimated fractions and their errors

Age	0-	20-	40-	60-
Fractions	0.05	0.14	0.13	0.22
	±0.001	±0.004	±0.003	±0.009

Table 8. Result of treatment of suppurative infections in infants and children aged up to 5 years

Disease category	No. of cases	Result of treatment			
		+	++	+++	++++
I	1			1	
VIII	6		1	4	1
IX	1				1
X	1			1	
XII	3			2	1
XIII	3			3	
XVIII	2			2	
	17		1	13	3

a transient improvement of the local state (+) and the average age of the completely cured patients (++++) was as high as 13.1. To check whether the relation between the effect of phage therapy and age observed in the study, is significant for the whole population, the use was made of the *t* test with right-hand critical region to compare the average age of not completely cured patients (group +, ++ with average age 41.6) with that of completely cured (group +++, ++++ with average age 32.2). The obtained value ($r = 1.81$, $p < 0.07$ with $r = 136$) confirms the statistical significance of the dependence between phage therapy and age of the patients.

Table 7 presents a number of patients with various effect of treatment: +, ++, +++, ++++ depending on age group. Patients from + and ++ groups were regarded not completely cured and from +++ and ++++ groups — completely cured. A question arose whether the number of not completely cured patients had a tendency to increase with age. Estimated fractions and their standard errors were calculated from the formula:

$$\sqrt{\frac{P_i q_i}{n_i}}.$$

A marked difference is observable between the extreem age groups i.e. 0—19 years and above 60. The former included only 5% of not cured patients and the latter — 22%. In the group 20—39 years and 40—59 years participation of the not cured patients ranged from 13 to 14%.

Of particular importance is a possibility of bacteriophage application in the treatment of intestinal and suppurative infections in infants and in 1—5-year-old children. The investigated group included 6 infants aged from 2.5 to 6 months and 11 children aged from 1 to 5 years. In all the cases phage therapy gave very good results. Full characteristics of these groups is depicted in Table 6.

The results obtained in the group of children up to 5 years old confirmed the earlier observed dependence between the age of patient and the result of phage therapy. In this age group as much as 95% of cases were completely cured. As follows from the statistical evaluations ($u = 3.39$, $p < 0.001$), such good results may be expected in all children up to 5 years old.

Group of infants from 2.5 to 10 months old

2*. A.S. ♀ 5 months old (Q41.1. 041.3)**	Burns of upper extremity, amputation of a finger, acute suppurative inflammation of the middle ear and septicemia; treatment with antibiotics ineffective. Phages were applied locally on the lesion for 8 weeks (moist application), as eye drops for 5 weeks and orally for 5 weeks; complete healing of the local lesions (+++).
50. K.K ♀ 7 months old (041.1)	Pneumonia, diarrhea; ineffective treatment with antibiotics; phages applied orally for 1 week (without simultaneous antibiotic administration), serious state, complete recovery from pneumonia and diarrhea (+++).
59. K.A. ♀ 2.5 months old (041.1 041.7)	Pneumonia, thoracic empyema treated ineffectively with antibiotics; phages administered orally for 4 weeks and applied locally (on the lesion, to the fistula and drainage of thoracic cavity) for a week; marked improvement (++).
72. M.D. ♂ 6 months old (041.1)	Suppurative skin inflammation, mucopurulent conjunctivitis; antibiotics ineffective; phages administered orally for 3 weeks and applied locally on the lesion for 1 week; complete healing of the local lesions (+++).
102. S.P. ♂ 4 months old	Furunculosis; antibiotic treatment ineffective; phage administered orally and locally for 3 weeks; complete recovery (++++).
111. S.E. ♀ 10 months old (041.1, 041.7)	Pneumonia, thoracic empyema; acute inflammation of the middle ear; treatment with antibiotics ineffective; phages administered orally for 4 weeks and applied locally (on the lesion and to the fistula) for 3 weeks; complete healing of the local lesions (+++).

Group of children aged from 1 to 5 years

5. B.E. ♀ 5 years old (041.1)	Acute osteitis, persistent postoperative fistula; treatment with antibiotics ineffective; phages administered orally for 16 weeks and applied locally on the lesion and to the fistula for 12 weeks; complete healing of the local lesions (+++). Simultaneous administration of antibiotics.

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* Case number.

** Explanations of the symbols:

003.0 infection induced by *Salmonella enteritidis*
004.0 ,, ,, *Shigella sonnei*
041.0 ,, ,, *Streptococcus*
041.1 ,, ,, *Staphylococcus*
041.3 ,, ,, *Klebsiella*
041.4 ,, ,, *Escherichia*
041.6 ,, ,, *Proteus*
041.7 ,, ,, *Pseudomonas*

6. B.A. ♂ 1.5 years old (041.1, 041.4, 041.6)	Acute osteitis, persistent post-operative fistula; treatment with antibiotics ineffective; phages administered orally (15 weeks) and locally applied (on the lesion and to the fistula) for 12 weeks; complete healing of the lesions (+++).
13. CH.P. ♂ 3 years old (041.7)	Urinary tract infection; ineffective treatment with antibiotics; phages administered orally for 2 weeks; complete sterilization of urinary tract (+++).
36. G.T. ♂ 4 years old (041.7)	Acute osteitis, persistent post-operative fistula; ineffective treatment with antibiotics; phages administered orally for 8 weeks; complete healing of the local lesions (+++).
43. M.M. ♀ 4 years old (041.1)	Mucopurulent bronchitis; ineffective treatment with antibiotics; phages administered orally for 4 weeks complete recovery (++++).
49. K.A. ♀ 1.5 years old (041.1)	Furunculosis of the face; ineffective treatment with antibiotics; phages administered orally for 5 weeks and applied locally (on the lesion and to the fistula) for 3 weeks; complete healing of the local lesions (+++)
52. K.A. ♀ 4 years old (041.7)	Acute pancreatitis (disturbances in absorption), pneumonia; ineffective treatment with antibiotics; phages administered orally for 3 weeks; complete recovery (+++).
53. K.E. ♀ 1.5 years old (004.3)	Dysentery; not treated with chemotherapeutics; phages administered orally for 1 week; complete recovery (++++).
62. K.S. ♂ 2.5 years old (041.4)	Pneumonia, thoracic empyema with fistula; ineffective treatment with antibiotics; serious state; phages administered orally for 2 weeks; complete recovery (+++).
82. P. K. ♀ 1.5 years old (041.3, 041.7)	Pneumonia, thoracic empyema with fistula, ineffective treatment with antibiotics; phages administered orally for 7 weeks and locally applied on the wound and to the fistula for 7 weeks; full recovery (+++).
137. Ż. P. ♂ 3 years old (041.1, 041.7)	Fracture of fornix of the scull with fistula and purulent subscleral posttraumatic hematoma, contusion of heel; ineffective treatment with antibiotics; phages applied orally and locally for 4 weeks; full recovery (+++).

Results of phage therapy and sex of patients

Result of phage therapy in relation to patients' sex is illustrated in Table 9. Statistical evaluation was based on the chi-square test for two-by-two contigence table with regard to frequencies (test 6).

Table 9. Sex of patients and result of bacteriophages treatment

Sex		Result of treatment				Total
		+	−+	++−	++++	
Females	No. of cases	4 6.6%	3 4.9%	43 70.5%	11 18%	61 44.2%
	AA	52.8	26.4	31.1	32.2	
	SD	8.9	20.1	22.5	20.8	
Males	No. of cases	5 6.5%	5 6.5%	56 72.7%	11 14.3%	77 55.8%
	AA	40.0	43.2	32.8	32.9	
	SD	18.3	19.0	18.2	19.4	

To test the above dependence, a comparison was made within the group of completely cured patients (++−++) since this group displayed most substantial difference in the effect of treatment between men and women (3.7%). However, no dependence between sex of patients and effect of phage therapy was noted ($\chi^2 = 0.36$, $p > 0.05$ with $r = 1$).

Results of phage therapy in mono- and polyinfections

Effectiveness of phage therapy in mono- and polyinfections is illustrated in Tables 10-15.

Table 10 presents a general comparison of phage therapy in relation to a type of infection (monoinfections, polyinfections), average age of patients and standard de-

Table 10. Result of phage therapy in mono- and polyinfections

Type of infection		Result of treatment				No. of cases
		+−	++−	+++	++++	
Monoinfection	No. of cases	5 5.5%	3 3.3%	67 74.4%	15 16.8%	90
	AA	33.3	42.0	31.8	30.7	
	SD	11.5	17.5	19.5	19.9	
Polyinfection	No. of cases	4 8.3%	5 10.4%	32 66.7%	7 14.6%	48
	AA	60.5	33.8	32.7	36.7	
	SD	5.5	22.4	21.7	20.0	

viation of age. Statistical evaluation of the results obtained was based on the chi-square test for two-by two contingence table with regard to frequencies (test 6).

The values of χ^2 test were calculated for all the degress of recovery ($\chi^2_{+} = 0.39$, $\chi^2_{++} = 2.18$, $\chi^2_{+++} = 0.89$, $\chi^2_{++++} = 0.16$).

The results obtained did not confirm the possible differences in the effect of phage therapy in mono- and polyinfections ($p > 0.05$).

Table 11 contains the data concerning results of phage therapy in 90 monoinfection cases in relation to a species of bacteria that induced the monoinfection. Due

Table 11. Monoinfections and result of treatment

Species of bacteria	Result of treatment				No. of cases
	+	++	+++	++++	
041.1 *Staphylococcus*	5	2	43	12	62 68.9%
041.3 *Klebsiella*	0	1	4	0	5 5.6%
041.4 *Escherichia*	0	0	8	1	9 10.0%
041.6 *Proteus*	0	0	0	0	0
041.7 *Pseudomonas*	0	0	12	0	12 13.3%
004.3 *Shigella*	0	0	0	1	1 2.2%
003.0 *Salmonella*	0	0	0	1	1
Number of cases	5	3	67	15	90
Average age (AA)	33.8	42.0	31.8	30.7	
Standard deviations of age (SD)	11.5	17.5	19.5	19.9	

to a high number of monoinfections induced by *Staphylococci* a question arose whether the majority of monoinfection is induced by *Staphylococci.* analysis was based on the test of inference on the basis of frequencies (test 3). The obtained u value ($u = 14.65$) allows to infere that most monoinfections is caused by *Staphylococci* ($p < 0.001$).

Another problem to solve was whether there is any difference in the effect of the monoinfection. Statistical analysis was based on the chi-square test for two-by-two contingence table with regard to frequencies (test 6). Considering two groups of patients: + and ++, +++ and ++++, no difference was observed in the effect of phage therapy of monoinfections induced by: *Staphylococcus* and *Pseudomonas*, *Klebsiella* and *Pseudomonas*, and also *Escherichia* and *Pseudomonas*. An example of

analysis of *Staphylococcus* and *Pseudomonas* infections looks as follows: χ^2 for 2×2 contingence table with regard to frequencies, r = 1 χ^2_{obs} 1.49 $\chi^2_{0.05} = 3.84$ $p > 0.05$.

There is no significant difference in the effect of phage therapy applied in monoinfection induced by *Staphylococcus* and *Pseudomonas*.

Table 12 contains data concerning a frequency of occurrence of particular bacterial species in polyinfections. In statistical analysis of the results obtained, two bacterial

Table 12. Polyinfections and result of treatment

Species of bacteria	Result of treatment				No. of cases
	+	++	+++	++++	
041.1 *Staphylococcus*	1	5	22	7	35 72.9%
041.3 *Klebsiella*	3	0	13	0	16 33.3%
041.4 *Escherichia*	3	1	10	2	16 33.3%
041.6 *Proteus*	2	0	10	2	14 29.2%
041.7 *Pseudomonas*	2	4	20	3	29 60.4%
041.0 *Streptococcus*	0	0	2	1	3 10.4%
041.8 Other species	0	0	1	1	2
No. of cases	4	5	32	7	48
Average age (AA)	60.5	33.8	32.7	36.7	
Standard deviation of age (SD)	5.5	22.4	21.7	20.0	

species (*Staphylococcus* and *Pseudomonas*), most frequently represented on the material investigated, were considered. Of interest was whether on the basis of the results obtained in a group of 138 cases, it may be inferred that among all polyinfections, majority is induced by the above bacteria occurring together or in combination with other microorganisms. Statistical analysis was based on the test of inference on the basis of frequencies (test 3).

The obtained results: a) for *Staphylococcus* $u = 11.3$, $p < 0.001$, b) for *Pseudomonas* $u = 8.9$, $p < 0.001$, confirmed that the inference was correct in both cases.

Another worth noting problem was whether the bacteria being a cause of monoinfections and occurring together with other bacteria (polyinfection) may bring a statistically significant difference in the effect of phage therapy. Statistical analysis of the joined groups + and ++, +++ and ++++ was based on the chi-square

test of independence between two variables with $r = 1$ (test 5). Effect of phage therapy in mono- and polyinfections was compared for four bacteria:

a) *Staphylococcus* ($\chi^2 = 0.56$, $p \gg 0.05$),
b) *Klebsiella* ($\chi^2 = 0.02$, $p \gg 0.05$),
c) *Escherichia* ($\chi^2 = 1.06$, $p > 0.05$),
d) *Pseudomonas* ($\chi^2 = 1.58$, $p > 0.05$).

For all the above organisms, no dependence between the type of infection and the result of treatment was observed.

Another problem of our concern was whether a given bacterial species occurs with equal frequency in mono- and polyinfections. Statistical analysis was based on the test of comparison of two frequencies from independent samples (test 4).

041.1 $u_{obs} = 049$ $u_{0.001} = 3.29$ $p \gg 0.05$

Staphylococcus (041.1) occurs equally frequently in mono- and polyinfection.

041.3 $u_{obs} = 4.23$ $u_{0.001} = 3.29$ $p < 0.001$

Klebsiella (041.3) occurs more frequently in polyinfection.

041.4 $u_{obs} = 3.35$ $u_{0.001} = 3.29$ $p < 0.001$

Escherichia (041.4) occurs more frequently in polyinfection.

041.7 $u_{obs} = 5.76$ $u_{0.001} = 3.29$ $p < 0.001$

Pseudomonas (041.7) occurs more frequently in polyinfection.

The influence of age and sex to the appearance of monoinfection and polyinfection was also subjected to statistical analysis (Tables 13 and 14). In the case of age

Table 13. Age of patients with mono- and polyinfections

Type of infection	Age	
	average age (AA)	standard deviation (SD)
Mono- n = 90	32.0	19.2
Poli- n = 48	35.7	21.9

Table 14. Sex and mono- and polyinfection

Sex	Type of infection		
	mono-	poli-	
Females	41	20	61
	67.2%	32.8%	
Males	49	28	77
	63.6%	36.4%	

the use was made of the t-test of the differences between two means from independent samples assuming that $\sigma_1^2 = \sigma_2^2$ and in the case of sex the chi-square test of independence between two variables (test 5) was applied.

In both cases no dependence between the investigated traits and infection type was noted (for age: $t = 1.03$, $p > 0.05$, for sex $\chi^2 = 0.19$, $p \gg 0.05$, with $r = 1$).

Result of phage therapy and severity of infection course

State of the patients at the beginning of phage therapy is presented in Table 15. Statistical analysis of the results obtained was based on the chi-square test of independence between two variables (test 5). No significant dependence between the result of treatment and patient's state at its beginning was noted ($\chi^2 = 1.47$, $p > 0.05$, with $r = 6$).

Table 15. Severity of the disease and result of the treatment

State of patient at the beginning of therapy		Result of treatment				No. of cases
		+	++	+++	++++	
Without disturbances in general state		4	3	41	9	57
		7.0%	5.3%	71.9%	15.8%	41.3%
	AA	46.0	34.3	32.4	38.4	
	SD	15.5	20.2	20.4	18.3	
Medium state		2	2	37	10	51
		3.9%	3.9%	72.6%	19.6%	37.0%
	AA	32.0	50.4	30.3	24.0	
	SD	16.0	27.1	20.5	18.6	
Severe state		3	3	21	3	30
		10%	10%	70%	10%	21.7%
	AA	54.3	30.4	34.3	43.7	
	SD	9.8	27.1	19.4	18.6	

Results of phage therapy and resistance to antibiotics

Phage therapy was performed on 125 patients (90.6%) in whom antibiotic treatment appeared ineffective and the bacteria showed resistance to most of the antibiotics applied (Table 16). Antibiotic-resistant group was compared with a small, not treated with antibiotics group of patients. It included 13 subjects (9.4%).

Statistical analysis was performed on the basis of the chi-square test of independence between two variables (Test 5).

$\chi^2_{obs} = 4.76$ - $\chi^2_{0.05} = 7.81$ for $r = k-1 = 3$ degrees of freedom $p > 0.05$

The result of treatment is not significantly related to whether antibiotic treatment was ineffective or not applied prior to phage therapy.

Among 138 bacteriophage treated cases, 125 were antibiotic-resistant (Table 17). An attempt was made to answer a question whether it may be inferred that an increase in the number of patients would bring about an increase of cases resistant to anti-

Table 16. Resistance to antibiotics and result of therapy

Antibiotic treatment before bacteriophage application		Result of treatment +	++	+++	++++	No. of cases
Ineffective		9 7.2%	6 4.8%	93 74.4%	17 13.6%	125
	AA	45.7	32.4	31.8	33.0	90.6%
	SD	16.2	20.7	20.3	20.1	
No treatment		0	2 15.3%	6 46.2%	6 38.5%	13
	AA		50.5	36.0	31.3	9.4%
	SD		15.5	17.6	20.3	

Table 17. Resistance to antibiotics and result of therapy

	Result of treatment +	++	+++	++++	Total
Cases treated with bacteriophages	9 6.5%	8 5.7%	99 71.7%	22 16.1%	138
Cases resistant to antibiotics and treated with bacteriophages	9 7.2%	6 4.8%	93 74.4%	17 13.6%	125

biotics. To this end the test of inference on the basis of frequencies (test 3) was applied.

$u_{obs} = 9.45 \quad u_{0.001} = 3.29, \quad p < 0.001$

Among all the patients the percentage of those resistant to antibiotics would be about 90%.

Applying the same test it was attempted to check whether among a great number of patients, the number of cured subjects with +++ and ++++ result would be as high as in the investigated group (110 cases cured with +++ and ++++ result and 125 cases resistant to antibiotics). According to the results of our calculations the positive effect of treatment can be also obtained among a great number of patients resistant to antibiotics ($u = 8.41$, $p < 0.001$).

Result of phage therapy in combination with antibiotics and without antibiotics

Table 18 compares the results of bacteriophage application in the combination with antibiotics and without them. It was decided to test whether phage therapy is more effective with or without antibiotics. Statistical analysis of the results obtained was based on the chi-square test with $r = 1$ (test 5).

Table 18. Bacteriophage treatment in combination with antibiotics and without antibiotics

Treatment		Result				No. of cases
		+	++	+++	++++	
Without antibiotics	No of patients	5	8	59	18	90
		5.5%	8.8%	65.5%	20.2%	
	AA	36.4	36.9	32.6	35.6	
	SD	14.2	21.1	20.6	21.0	
With antibiotics	No. of patients	4	0	40	4	48
		8.3%		83.4%	8.3%	
	AA	57.3	0	31.3	19.0	
	SD	9.9	0	19.5	4.5	

Group cured with ++ result:

$\chi^2_{obs} = 3.08 \quad \chi^2_{0.05} = 3.84, \quad p > 0.05.$

The difference observed is statistically nonsignificant.

Group cured with +++ result:

$\chi^2_{obs} = 4.84 \quad \chi^2_{0.05} = 3.84, \quad p < 0.05.$

The difference observed (to the advantage of the treatment with antibiotics) is statistically significant.

Group cured with ++++ result:

$\chi^2_{obs} = 3.25 \quad \chi^2_{0.05} = 3.84, \quad p > 0.05.$

The difference observed (to the advantage of the treatment without antibiotics) is statistically significant.

Therefore, it cannot be unequivocally stated that phage therapy is more effective in the combination with antibiotics or without them since the differences observed in its effect vary in particular categories of curing.

Result of phage therapy in relation of the route of phage administration

Of substantial importance in phage therapy is the route of phage administration. In the examined group of 138 patients phages were applied orally and locally (109 cases), orally only (22 cases) and locally only (7 cases). Locally, they were used as moist applications and in some cases as flow drainage. General result of phage therapy when the three above routes were applied is presented in Table 19.

The Table illustrates the differences related to the mode of phage therapy application. It was of interest to test whether they were significant enough to confirm the concrete dependence between therapy mode and treatment effect. Statistical analysis of the results was based on the chi-square test with 6 degrees of freedom (test 5).

$\chi^2_{obs} = 7.42 \quad \chi^2_{0.05} = 12.59, \quad p > 0.05$

The differences resulting from various modes of phage therapy are statistically nonsignificant.

Table 19. Result of phage therapy in relation to bacteriophage administration

Phage therapy	No. of cases	Result of therapy			
		+	++	+++	++++
local only	7	0	0	4 60%	3 40%
oral only	22	2 9%	2 9%	11 50%	7 32%
local and oral	109	7 6.4%	6 5.6%	84 77%	12 11%
No. of cases	138	9	8	99	22

In the group of patients treated with +-+-++ result a great span between the effect of the local and the local-oral phage therapy was observed. However, the scarce number of the subgroups did not allow to draw general conclusion.

Oral administration of bacteriophage. In 22 cases bacteriophages were administered orally only. The result of oral phage therapy is illustrated in Table 20.

Detailed analysis of the cases treated orally is as follows:

Table 20. Oral phage therapy

No. of cases	Result of treatment			
	+	++	+++	++++
22*	2	2	11	7
131*	9	8	95	19
	6.9%	6.1%	72.5%	14.5%

* Cases treated orally only
** Cases treated orally and locally

Oral phage therapy

53. K.E. ♀ 1.5 years old (004.3) — Dysentery (*Sh. sonnei*), treated orally with phage for a week; intermediate state; complete recovery without relapse (++++).

79. N.K. ♀ 44 years old (003.0) — Salmonellosis (*S. enteritidis*) treated orally with phage for 3 weeks; intermediate state; complete recovery without relapse (++++).

89. P.Z. ♀ 53 years old (041.1) — Varicose ulcer of veins of lower extremities; resistant to antibiotics; treated with phage orally (4 weeks); complete healing of the local lesions (+++).

27. D.T. ♀ 30 years old (041.1, 041.4) — Pneumonia, long-term glomerulonephritis, immunological disorders, not treated with antibiotics; phages administered orally for 1.5 weeks without antibiotics; full recovery (++++).

43. H.M. ♀ 43 years old (041.1)	Mucopurulent bronchitis, resistant to antibiotics; phage administered orally for 4 weeks without antibiotics; full recovery (++++).
50. K.K. ♀ 7 months old (041.7)	Pneumonia, functional diarrhea; resistant toantibiotics; phage administered orally for 1 week; healing of the lungs lesion (+++).
61. K.T. ♀ 30 years old (041.1)	Chronic pharyngitis, disorders of humoral immunity, resistant to antibiotics; phage administered orally for 3 weeks; complete recovery (++++).
62. K.S. ♀ 2.5 years old (041.7)	Pneumonia, thoracic empyema with a fistula, resistant to antibiotics; phage administered orally for 2 weeks; healing of the thoracic lesion (+++).
65. L.S. ♀ 30 years old (041.1, 041.7)	Bronchiectasis, mucopurulent bronchitis, resistant to antibiotics; phages administered orally for 3 weeks; marked improvement (++).
83. P.T. ♂ 48 years old (041.1, 041.6)	Bronchogenic pneumonia, chronic glomerulonephritis, not treated with antibiotics; phage administered orally for 3 weeks; recovery (+++).
114. T.G. ♀ 48 years old (041.1)	Chronic pansinusitis, chronic tonsillitis, allergic rhinitis; resistant to antibiotics; phage administered orally for 12 weeks; full recovery (++++).
52. K.A. ♀ 4 years (041.7)	Pneumonia and acute pancreatitis, postoperative disturbances in absorption, resistant to antibiotics; phage administered orally for 3 weeks; healing of the local lesions (+++).
13. CH. P. ♂ 3 years old (041.7)	Urinary tract infection, resistant to antibiotics; phage administered orally for 3 weeks; recovery (+++).
118. T.A. ♀ 7 years old (041.4)	Urinary tract infection, resistant to antibiotics; phage administered orally for 4 weeks; recovery (+++).
24. D.Z. ♂ 32 years old (041.7)	Suppurative skin inflammation, resistant to antibiotics; phage administered orally for 3 weeks; recovery (+++).
112. S.D. ♂ 12 years old (041.1, 041.4)	Furunculosis of the face, resistant to antibiotics; phages administered orally for 6 weeks in combination with antibiotics; complete recovery (++++).
129. W.P. ♂ 25 years old (041.1)	Acute ostitis, not treated with antibiotics; phage administered orally for 1 week; recovery (+++).
110. S.K. ♀ 20 years old (041.1)	Arteriostenosis — surgical treatment, acute suppurative pericarditis thoracic empyema with a fistula, resistant to antibiotics; phage administered orally for 3 weeks in combination with antibiotics; recovery (+++).
4. B. M. ♂ 66 years old (041.3)	Mucopurulent bronchitis, bronchial asthma not treated with antibiotics; phage administered orally for 5 weeks without antibiotics; marked improvement of the local state (++).

29. F.Z. ♀ 24 years old (041.7) — Phlebitis of lower extremity, pneumonia, chronic glomerulonephritis, resistant to antibiotics; phage administered orally for 4 weeks (without antibiotics); recovery (++++).

47. K.K. ♂ 55 years old (041.4, 041.6, 041.7) — Pneumonia, bronchiectasis, treated ineffectively with antibiotics; phages administered orally for 6.5 weeks, transient improvement of the local state (+); (case difficult for analysis due to the additional infection with *Aspergillus fumigatus*).

136. Ż.P. ♀ 42 years old (041.1) — Pneumonia, long-term glomerulonephritis, sepsis, antibiotics — ineffective; phage a — administered orally for 2 weeks together with antibiotics; improvement of the local state (+); general state — serious, cachexia, edema of lungs and brain, exit.

The results obtained confirmed great effectiveness of this mode of phage therapy, particularly in bacterial infections of the digestive system, diseases of the respiratory system (bacterial pneumonia) and even in the skin diseases and suppurative infections of the urinary tract.

Statistical analysis of the results of oral therapy revealed that the markedly positive effect of oral administration of bacteriophages (82% of patients cured with +++ and ++++ result) can be also obtained in a numerous group of patients receiving bacteriophages orally only (test 3, $u = 5.93$, $p < 0.001$).

Local application of bacteriophage. In 7 cases bacteriophage was applied locally only. The results are depicted in Table 21. Though the number of cases was rather

Table 21. Local phage therapy

No. of cases	Result of treatment			
	+	++	+++	++++
7*			4	3
116**	7	6	88	15
	6.0%	5.2%	75.9%	12.9%

* Cases treated locally only
** Cases treated locally and orally

small, the effect is very encouraging. This mode of treatment may find its application in the healing of burns complicated by bacterial infections, suppurative diseases of the upper respiratory tract, particularly of pansinusitis and maxillar sinusitis as well as in the diseases of conjunctival sac, middle ear and skin furunculosis.

Flow drainage

1. A.A. ♀ 10 years old (041.3, 041.4, 041.6) — Pyogenic arthritis, sepsis, chronic osteomyelitis; ineffective treatment with antibiotics; phages administered

041.7)	orally (26 weeks) and applied locally on the wound and to the fistula, flow drainage; recovery (+++).
26. D.F. ♂ 43 years old (041.1)	Chronic maxillar sinusitis, antibiotics — ineffective, phages administered orally for 2 weeks and applied locally (flow drainage); recovery (+++).
45. J.T. ♂ 49 years old (041.4)	Gastric ulcer with perforation, suppurative peritonitis (postoperative); antibiotics were not given; phages given orally for 3 weeks and locally on the lesion, to the fistula and as flow drainage to the peritoneal cavity (3 weeks); recovery (+++).
59. K.A. ♀ 2.5 months old (041.1, 041.7)	Thoracic empyema with a fistula, pneumonia; antibiotics ineffective phages given orally for 4 weeks and locally applied on the wound and to the fistula as flow drainage. Marked improvement (+-).
67. M.Z. ♀ 12 years old (041.1, 041.0, 041.7)	Chronic osteitis, suppurative myositis, fistula, sepsis; antibiotics — ineffective phage administered orally for 13 weeks and locally on the lesion, to the fistula and as flow drainage; recovery (+++).
73. M.W. ♀ 47 years old (041.1, 041.3)	Mucopurulent bronchitis, thoracic empyema with a fistula. Opening of the wound after operation of esophagus cancer antibiotics — ineffective; phages administered orally (7 weeks) and applied locally (4 weeks) on the wound and to the fistula andas flow drainage; recovery (+++).
92. R.W. ♂ 52 years old (041.3, 041.4)	Acute inflammation of the gall-bladder, acute pancreatitis, suppurative-peritonitis bronchopneumonia. Antibiotic treatment ineffective; phages administered orally (5 weeks) and locally (1 week) applied on the wound, to the fistula and as a drainage; recovery (+++).
95. S.M. ♀ 53 years old (041.7)	Urinary tract infection, antibiotics — ineffective, phages administered orally (4 weeks) phage-lavage of the urinary bladder (1 week); recovery (+++).

Result of phage therapy and period of phage administration

The period of phage administration with regard to the route of administration and the number of patients is presented in Table 22. As seen, most of the cases were treated during the period from 2 to 15 weeks; percentage of cases treated orally was 88.1% (96 cases) and that of locally treated — 82.6% (90 cases).

Effect of treatment in relation to the oral or local application of bacteriophages is illustrated in Table 23. From the Table it follows that at oral administration of bacteriophage, the effect of treatment is conditioned rather by other factors than the duration period. It was well evidenced by the cases with the transient improvement of the local state (+) with the longest period of treatment.

Table 22. Period of phage administration with regard to the route of administration and numbr of cases treated

Period of phage administration	Route of phage administration			
	orally + locally		only orally	only locally
1 week	0	7	3	
1,5 weeks	5	4	1	
2.0 weeks	14	16	3	3
—5 weeks	41	41	12	3
36—10 weeks	33	29	2	
11—15 weeks	8	4	1	1
16—20 weeks	5	5		
20 weeks	3	3		
No. of cases	109		22	7
	79.0%		15.9%	5.1%

Table 23. Significance of the period of bacteriophage administration (the mean does not include the patients being treated by other than local or oral route)

Period of bacteriophage administration in weeks		Result of treatment			
		+	++	+++	++++
Orally	Mean	7.3 ±4.9	4.6 ±2.3	6.4 ±4.8	5.8 ±5.1
	AA	45.7	36.9	31.4	29.3
	SD	16.2	21.1		
Locally	Mean	4.6 ±1.9	3.5 ±1.4	5.6 ±4.7	5.7 ±4.1
	AA	44.9	33.2	34.2	36.5
	SD				

At local application, however, the tendency toward better therapy effect was growing with the duration of phage administration. The cases difficult for analysis were the exception.

The observed tendency has not yet found a statistical confirmation as manifested by the results of Wilcoxon test (test 7) (e.g. for ++ adn +++ classes, $W = 62$, $p > 0.05$).

Detailed analysis of the values obtained in the above study is presented in Part II.

Result of phage therapy in particular disease categories

Table 24 illustrates the results of bacteriophage treatment in particular disease categories. The aim of the statistical analysis was to test whether there exists a de-

Table 24. Result of treatment in particular disease categories

Category	No. of cases	Result of treatment				
		+	++	+++		++++
I	2					2
III	2					2
VII	8		1	5	85.7%	2
VIII	17	3	4	8	58.8%	2
IX	17	1		15	94.1%	1
X	6			6		
XII	20	1		14	95%	5
XIII	31	3	0	24	90.3%	4
XVII	35	1	3	27	88.5%	4
No. of cases	138	9	8	99		22

pendence between the result of the treatment and category of the disease. To answer this question, patients cured with + and ++ result were joined together to form one group; the same concerned patients cured with +++ and ++++ result. Patients with diseases of VIII category appeared to have poor chance of recovery (+++, ++++) (58.8%), the best chance (excluding categories of diseases of a number $\leqslant 7$) had patients with the following disease categories:

XII 95%, IX 94%, XVII 88.5%, XIII 90.3%

Category VIII was compared with all the above categories and the values of χ^2 test with $r = 1$ were calculated (test 5).

1. VIII and XII, $\chi^2_{obs} = 5.03$ $\chi^2_{0.05} = 3.84$, $p < 0.05$
2. VIII and IX, $\chi^2_{obs} = 3.22$ $\chi^2_{0.05} = 3.84$, $p > 0.05$
3. VIII and XVII, $\chi^2_{obs} = 4.14$ $\chi^2_{0.05} = 3.84$, $p < 0.05$
4. VIII and XIII, $\chi^2_{obs} = 4.72$ $\chi^2_{0.05} = 3.84$, $p > 0.05$

The results obtained revealed that the differences in phage therapy effect between categories VIII and categories XII, XIII, XVII are statistically significant ($p < 0.05$). From the above it follows that patients with VIII category of disease have poorer chances of recovery than those with XII, XVII and XIII categories. Can it be, thus, inferred that among all the patients with XII, XIII and XVII categories a percentage of the cured would be as high?

To this end the values of "u" variable were calculated (test 3)

XII: $u_{obs} = 3.79$ $u_{0.001} = 3.29$ $p < 0.001$
XIII: $u_{obs} = 4.31$ $u_{0.001} = 3.29$ $p < 0.001$
XVII: $u_{obs} = 4.71$ $u_{0.001} = 3.29$ $p < 0.001$

The findings of the calculations allow the possibility that among all patients with XII, XIII and XVII categories of disease, the percentage of cases cured with +++ and ++++ result can be as high as in Table 24 (XII — 95%, XIII — 90.3%, XVII — 88.5%).

Side effects of bacteriophage treatment

Side effects in the course of phage therapy are very rare (Table 25). Out of 13 treated subjects, only 3 cases with side effects were recorded of which 2 displayed drug intolerance at oral administration and 1 allergic symptoms at local application

Table 25. Side effects after phage therapy

	Occurred	Did not occur
No. of cases	3 2.2%	135 97.8%
AA (average age)	24.3	33.2
SD (standard deviation)	4.9	20.1

on the wound. Statistical analysis was based on the test of inference on the basis of frequencies (test 3).

$u_{obs} = 11.16 \quad u_{0.001} = 3.29, \quad p < 0.001$

With the increased number of patients, the number of subjects without side symptoms would be as high as in the cases observed.

On the basis of case history the following observations were made: mostly on day 3 — 5 of phage therapy, hepatalgia occurred which lasted several hours. This can be accounted for mass liberation of endotoxins resulting from phage effect on bacteria. In severe cases with sepsis, an increase of temperature occurred on day 7 — 8 of phage administration which lasted 24 h.

Therefore, up to the 8th day of treatment with bacteriophages, the patients should be closely observed. Generally, on the 7th day marked improvement in the general feeling of patients was observed, the pains subsided, and the temperature and ESR fell down.

Local application of phage directly on a wound or to a fistuls caused a purification of a wound on day 3 — 4 and also exceptionally quick granulation.

DISCUSSION

In the available literature we have not encountered any reports concerned conditions of therapeutic effect of bacteriophages. The present report is an attempt of analysis of the dependence between phage therapy effect and various factors such as: age, sex, infection type (monoinfections, polyinfections), bacterial species inducing the suppurative process, severity of disease course, susceptibility and resistance to antibiotic and chemotherapeutic treatment, combined treatment with antibiotics, route and period of phage administration disease category and side effects.

Since phages are applied mostly when all ways of treatment appeared ineffective, thus, the criteria used for evaluation of the results, in majority of cases, cannot be

preserved. Besides, the state of patients treated with bacteriophages as the only effective remedy is often serious, even agonal. Thus, in spite of a markedly favorable effect of phage therapy (healing of the local wounds), due to the advanced anatomopathological changes of the internal organs, a death follows.

The results obtained so far showed that bacteriophages are a valuable and often the only effective factor in the bacterial infections.

— Bacteriophages are safe, side effects are rather rare and present no danger for a patient, they are transient and easy for restraint.

— Bacteriophages effectively control the infectious process, irrespectively of its localization, rebuilding the immunity forces of an organism. They are effective in the treatment of infections complicating immunological disorders.

— Bacteriophages can be administered orally or applied locally, they easily penetrate from the gastrointestinal tract to the blood, body fluids and urine exerting substantial therapeutic effect. It is of particular importance in the cases when they cannot be used directly.

— Bacteriophages can be used irrespectively of age, results of the treatment of suppurative infection in infants and small children are very promissing.

— Bacteriophages can be prepared at appropriate laboratories, individually for each case which ensures reliability of their effect.

— Worth noting is also a low cost of their preparation.

Phage therapy undoubtedly deserves a special attention as an important and effective factor in the treatment of suppurative infections resistant to antibiotics and chemotherapeutics.

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RESULTS OF BACTERIOPHAGE TREATMENT OF SUPPURATIVE BACTERIAL INFECTIONS IN THE YEARS 1981–1986*

by

STEFAN ŚLOPEK, BEATA WEBER-DĄBROWSKA, MAREK DĄBROWSKI and ALINA KUCHAREWICZ-KRUKOWSKA

Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Czerska 12, 53-114 Wrocław

In the years 1981–1986 bacteriophage therapy was applied in 550 cases (100 treated in 1986) of suppurative bacterial infections. Positive results were obtained in 508 cases (92.4%). In 38 cases (6.9%) a transient improvement was observed and in 4 cases (0.7%) phage treatment proved ineffective. Considering that majority of patients (518 cases, 94.2%) were resistant to antibiotic treatment, the results of phage therapy may be regarded as favorable.

Previous reports[1–6] presented the results of phage treatment carried out in 1981–1984 (370 cases). Since the results obtained in the group of further 180 cases of 1985 and 1986 did not diverge from those previously published, it was decided to sum up the whole material in the present report.

MATERIALS AND METHODS

Bacteriophage treatment was applied in 550 patients with septic bacterial infections caused by pyogenic *Staphylococci* (*Staphylococcus*) and Gram-negative bacteria (*Klebsiella, Escherichia, Proteus, Pseudomonas*). They were either spontaneous or postoperative complications.

Phage therapy was carried out at clinical or hospital departments, mostly surgery departments. The clinical results of phage therapy were evaluated by a therapeutist.

The age of patients ranged from 1 week to 86 years. Of 550 cases examined, the group of children up to 8 years constituted 76 cases (13.8%), group of children and teenagers in the age 9–21, 72 cases (13.1%), patients aged 21–60 years, predominating, 343 cases – (62.4%) and the smallest groups of patients above 60 years numbered 59 cases (10.7%).

The number of males – 288 cases (52.4%) was slightly higher than that of females – 262 cases (47.6%).

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The number of monoinfections (372 cases – 67.6%) was markedly predominating over polyinfections (178 cases – 32.4%). Most numerous were *Staphylococcal* monoinfections (272 cases – 73.1%), less frequent were monoinfections caused by Gram-negative bacilli (100 cases – 26.9%). In 151 polyinfection cases, pyogenic *Staphylococci* (121 cases – 68.0%) were predominating over Gram-negative bacilli (57 cases – 32.0%).

Phage therapy without parallel antibiotic therapy was applied in 398 cases (72.4%) and parallel administration of bacteriophages and antibiotics in 152 cases (27.6%). The number of cases resistant to antibiotics available amounted to 518 (94.2%).

In phage therapy the use was made of virulent bacteriophages, i.e. inducing a complete lysis of bacterial strains isolated from patients. Bacteriophages were administered orally 3 times daily in the dose of 10 ml before the meal, after previous neutralization of gastric juice. Phages were also used locally as moist applications to pleural, peritoneal, cavities, urinary bladder and as eye, ear and nose drops. In the course of treatment, sensitivity of isolated bacteria to phages applied was under control; in the case of confirmed resistance, bacteriophages were changed.

The final evaluation of the treatment results was based upon four-degree scale:

++++ outstanding effect manifesting by a complete recovery,
+++ elimination of suppurative process and healing of the local wounds,
++ marked improvement with a tendency to healing of the local lesions with negative results of bacteriological control,
+ transient improvement,
0 no effect.

RESULTS

While evaluating the final results, it should be taken into consideration that in 518 cases, i.e. in 94.2% the treatment preceding phage therapy failed, among others, due to resistance of bacteria to antibiotics and chemotherapeutics used. Thus, the application of bacteriophages was the only way to eliminate the pyogenic process. Only in 31 cases (5.6%) bacteriophages were used in patients not pretreated with either antibiotics or chemotherapeutics.

I. GENERAL CHARACTERISTICS OF PHAGE THERAPY

1. Age of patients and results of treatment

Detailed information is given in Table 1. Differences between the age groups up to 60 years and the group of patients above 60 years, achieved

Table 1. Age of patients and results of phage therapy

Age of patients	Results of treatment					No. of cases	No. of positive cases	Percent of positive cases
	0	+	++	+++	++++			
< 8 years		4	4	41	27	76	72	94.7
9–21 years	1	4	8	43	16	72	67	93.1
21–60 years	1	21	24	212	85	343	321	93.6
> 60 years	2	9	7	31	10	59	48	81.4
	4	38	43	327	138	550	508	92.4

1–4–X^2 = 6.02, ($p < 0.025$); 2–4–X^2 = 4.14, ($p < 0.05$); 3–4–X^2 = 9.99, ($p < 0.01$)

marked statistical significance. It is very likely that the above may be accounted for a reduced immunity of patients above 60 years of age.

2. Sex of patients and results of phage therapy

Phage therapy results in relation to the sex of patients are illustrated in Table 2. The differences observed were statistically significant.

Table 2. Sex of patients and results of phage therapy

Sex of patients	Results of treatment					No. of cases	No. of positive cases	Percent of positive cases
	0	+	++	+++	++++			
Female	2	11	20	159	70	262	249	95.0
Male	2	27	23	168	68	288	259	89.9
	4	38	43	327	138	550	508	92.4

$1-2-X^2 = 5.07$, $(p < 0.025)$

3. Type of infection (monoinfections, polyinfections) and results of phage therapy

Detailed data are given in Table 3. The results obtained in both types of infections reveal statistical significance.

Table 3. Results of phage therapy in monoinfections and polyinfections

Type of infection	Results of treatment					No. of cases	No. of positive cases	Percent of positive cases
	0	+	++	+++	++++			
Monoinfections	1	21	26	225	99	372	350	94.1
Polyinfections	3	17	17	102	39	178	158	88.8
	4	38	43	327	138	550	508	92.4

$1-2-X^2 = 4.83$, $(p < 0.05)$

4. Results of phage therapy in infections caused by pyogenic Staphylococci and Gram-negative bacteria

The data presented in Table 4 show that in infections caused by *Staphylococci* no statistical differences were observed between monoinfections and polyinfections. However, statistically significantly better results were obtained in Gram-negative bacteria-induced infections.

5. Method of treatment and result of phage therapy

Detailed data are given in Table 5. Statistically significant differences in favor of phage therapy in comparison to the parallelly run phage and antibiotic therapy, were confirmed.

Table 4. Effectiveness of phage therapy in monoinfections and polyinfections caused by pyogenes *Staphylococci* and Gram-negative bacteria

Etiology		Results of treatment					No. of cases	No. of positive cases	Percent of positive cases
		0	+	++	+++	++++			
Staphylococcus	M	1	15	20	157	79	272	256	94.1
Gram-negative bacteria	M		6	6	68	20	100	94	94.0
Staphylococcus	P	3	8	14	71	25	121	110	90.9
Gram-negative bacteria	P		9	3	31	14	57	48	84.2
		4	38	43	327	138	550	508	92.4

M — monoinfections
P — polyinfections
3—4—$X^2 = 1.74$, ($p < 0.25$), insignificant

Table 5. Results of phage therapy without and with parallel administration of antibiotics

Method of treatment	Results of treatment					No. of cases	No. of positive cases	Percent of positive cases
	0	+	++	+++	++++			
Phage therapy	1	18	28	240	111	398	379	95.2
Phage and antibiotic therapy	3	20	15	87	27	152	129	84.9
	4	38	43	327	138	550	508	92.4

1—2—$X^2 = 16.73$, ($p < 0.001$)

6. *State of patients and result of phage therapy*

The results presented in Table 6 suggest close relation between severity of disease and result of treatment. This problem with particular regard to the immunity state, advancement of the disease and results of phage therapy, requires further elucidation.

Table 6. Results of phage therapy in relation to a severity of patients status

Status of patient	Results of treatment					No. of cases	No. of positive cases	Percent of positive cases
	0	+	++	+++	++++			
Generally good	1	12	17	168	76	274	261	95.3
Medium-severe	2	10	12	99	34	157	145	92.4
Severe	1	16	14	60	28	119	102	85.7
	4	38	43	327	138	550	508	92.4

1—3—$X^2 = 10.71$, ($p < 0.01$); 2—3—$X^2 = 3.18$, ($p < 0.1$), insignificant

II. DETAILED CHARACTERISTICS OF PHAGE THERAPY APPLIED IN PARTICULAR DISEASE CATEGORIES

Classification into categories and nosologic units has been based on the recommendations of the World Health Organization included in the publication: Manual of the International Statistical Classification of Diseases, Injuries and Cause of Death, Vol. 1 WHO, Geneva, 1977. Detailed data are listed in Table 7.

Table 7. List of disorders as divided into disease categories with regard to nosologic units, monoinfections and polyinfections

Categories and nosologic units
I. Infections diseases
1. Infections diseases of the alimentary tract
2. Septicaemias
VI. Diseases of the nervous system and sense organs
3. Conjunctivitis, blepharoconjunctivitis, otitis media
4. Meningitis
VII. Diseases of the circulatory system
5. Varicose veins with ulcer and inflammation
6. Purulent pericarditis
VIII. Diseases of the respiratory system
7. Inflammation of the upper and lower respiratory tract
8. Pneumonia, pneumonia abscedens, bronchopneumonia
9. Suppurative pneumonia, empyema with thoracic fistula
10. Pleuritis with fistula
IX. Diseases of the digestive system
11. Ulcerative stomatitis. Suppurative peritonitis, abscesses of the inner organs
X. Diseases of the genitourinary tract
12. Suppurative infections of the urinary tract. Suppurative vaginitis
XII. Diseases of the skin and subcutaneous tissue
13. Furunculosis
14. Inflammation of the connective tissue and lymphatic vessels
15. Decubitus ulcer
XIII. Diseases of the musculoskeletal system
16. Pyogenic arthitis and myositis
17. Pyogenic ostitis
XVII. Injuries
18. Open wounds, laparotomy, tracheostomy, contusions, burns
19. Contusion of head, fractures of facial bones, contusion of brain and brain stem (suppuration of postoperative wounds)
20. Injuries of spine (suppuration of postoperative wounds)
21. Posttraumatic ostitis of the lower and upper limbs (fractures)
22. Chronic suppurative fistulas

Category I – 1 Infectious diseases of the alimentary tract

Three cases of bacillary dysentery, 2 cases of colibacteriosis, 1 case of *Salmonellosis* and 1 case of acute diarrhoea due to mixed infection with *Staphylococci, Klebsiella bacilli* and *Escherichia*, were submitted to phage

Table 8. Results of phage therapy applied in particular disease categories and noslogic units

For explanation see Table 7		Results of treatment 0	+	++	+++	++++	No. of cases	No. of cases	No. of positive cases	Percent of positive cases
1		2	3	4	5	6	7	8	9	10
I-1	M					6	6	7	7	100
	P					1	1			
I-2	M		5	7	29	7	48	98	87	88.8
	P		6	6	20	18	50			
VI-3	M				9	4	13	16	15	93.8
	P		1		2		3			
VI-4	M				2	1	3	10	9	90.0
	P	1		2	4		7			
VII-5	M		4	2	13	4	23	36	27	75
	P		5	2	5	1	13			
VII-6	M			1	3	1	5	7	7	100
	P				1	1	2			
VIII-7	M		2	10	30	11	53	74	67	90.5
	P	1	4	2	10	4	21			
VIII-8	M		1	2	13	4	20	57	47	82.5
	P	1	8	3	16	9	37			
VIII-9	M	1		1	7	2	11	27	23	85.2
	P		3	11	2		16			
VIII-10	M		3	1	7	2	13	22	19	86.4
	P			2	5	2	9			
IX-11	M		3	2	27	6	38	65	59	90.8
	P	1	2	2	17	5	27			
X-12	M			1	12	5	18	42	39	92.9
	P		3		13	8	24			
XII-13	M			2	19	29	50	55	55	100
	P				3	2	5			
XII-14	M		3	3	47	21	74	91	86	94.5
	P		2	1	9	5	17			
XII-15	M				2		2	16	13	81.3
	P		3		9	2	14			
XIII-16	M		1	1	9	3	14	19	17	89.5
	P		1		3	1	5			

1		2	3	4	5	6	7	8	9	10
XIII-17	M		2	3	19	5	29	40	36	90
	P				10	1	11			
XVII-18	M	1	1		13	7	22	49	42	85.7
	P		5	4	12	6	27			
XVII-19	M		3		10		13	23	19	82.6
	P		1	2	6	1	10			
XVII-20	M		1		4	3	8	12	11	91.7
	P			1	2	1	4			
XVII-21	M		3	1	18	2	24	41	37	90.2
	P		1	4	11	1	17			
XVII-22	M		6	4	87	21	118	180	168	93.3
	P		6	6	38	12	62			
								987	890	90.2

M — monoinfections
P — polyinfections

therapy. In all the 7 cases oral administration of bacteriophages for 1 to 3 weeks, resulted in a subsidence of the clinical symptoms and elimination of the infection process.

Category I — 2 Septicaemia

Septicaemia was confirmed in 98 of 550, i.e. in 17.8% cases submitted to phage therapy. The cases belonged to various disease categories. Monoinfections were recognized in 48 cases and polyinfections in 50. In monoinfections septicaemia was caused by in majority of cases by pyogenic *Staphylococci* (32 cases), less frequently by Gram-negative bacteria (*Klebsiella* — 5, *Escherichia* — 5, *Pseudomonas* — 6). In polyinfections most frequent appeared *Klebsiella* (37 cases), pyogenic *Staphylococci* (30 cases), *Escherichia* (30 cases), *Pseudomonas* (26 cases) and less frequent were *Proteus* bacteria (10 cases).

Positive therapeutic result was obtained in 87 cases (88.8%) and transient improvement in 7 cases. In 4 cases which ended with exitus, bacteriophages appeared ineffective (too late application at great cachexy of patients).

Category VI — 3 Blepharitis, conjunctivitis and otitis media

This group numbered 16 patients: 7 with purulent conjunctivitis, 3 with recurrent hordeolum, 1 with dacryocystitis and 5 with otitis media. All the

cases proved resistant to antibiotics. Pyogenic *Staphylococci* were isolated in monoinfections (13 cases), pyogenic *Staphylococci* and *Klebsiella* bacteria in 3 cases of polyinfections and *Pseudomonas* and *Proteus* bacteria in 1 case each. Application of bacteriophages as eye and ear drops and moist applications fully eliminated the infection.

Category VI — Meningitis

This group comprised 10 cases. In 9 patients meningitis resulted from operative infections and in 1 from septicaemia. All the cases were resistent to antibiotic treatment. Of 3 monoinfection cases, in 1 pyogenic *Staphylococci* was confirmed and in 2 *Klebsiella* bacilli. Klebsiella bacilli were predominating in polyinfections (6 cases of 7 cases), also frequent were pyogenic *Staphylococci* (5 cases); *Escherichia* and *Proteus* were encountered only in 2 cases each. In 9 cases phage therapy gave way to an elimination of pyogenic process. In 1 case due to the mixed infection with *Klebsiella*, *Escherichia* and *Pseudomonas*, exitus was reported.

Category VII — 5 Varicose veins of legs

This group included 36 patients. Of 23 monoinfection cases, 15 were caused by pyogenic *Staphylococci*, 7 by *Pseudomonas* and 1 case by *Klebsiella* bacteria. Of 13 polyinfections cases, *Pseudomonas* were confirmed in 7 cases, pyogenic *Staphylococci* in 4 cases and *Escherichia* in 1 case. All the cases were resistant to antibiotics. In 27 cases phage therapy resulted in a total elimination of the infection and in 9 marked improvement with tendency towards healing was confirmed.

Category VII — 6 Acute purulent pericarditis

This groups comparised 7 cases of pyopericardium (complications after operations on open heart and large vessels). In monoinfections (5 cases) pyogenic *Staphylococci* was recognized as a causative factor and polyinfections (2 cases) were caused by *Pseudomonas*, *Escherichia* and pyogenic *Staphylococci*. In all the cases antibiotic treatment brought no effect. In 6 cases phage therapy eliminated the infection and in 1 marked improvement was observed with a tendency towards healing of the wounds.

Category VIII — 7 Suppurative inflammation of the respiratory tract

Of 74 cases classified to this group, 51 patients suffered from suppurative inflammation of the nasal cavity, rhinitis, recurrent angina and 23 patients from mucopurulent bronchitis. Monoinfections were confirmed in 53 cases of which the majority was caused by pyogenic *Staphylococci* (46 cases) and a few

cases by *Escherichia* (4 cases) *Klebsiella* bacilli (2 cases) and *Proteus* (1 case). In 21 cases of polyinfections, most frequent were pyogenic *Staphylococci* (15 cases), *Klebsiella* bacilli (14 cases), less frequent were *Escherichia* (7 cases), *Pseudomonas* (6 cases), *Proteus* (2 cases) and *Streptococcus viridans* (1 case). Of 74 cases, in 69 antibiotic treatment failed. In 4 cases phage therapy was not preceded by antibiotic application. Phages appeared ineffective in 1 case, transient improvement was obtained in 6 cases and in the remaining 67 (91.9%) the suppurative process was fully eliminated.

Category VIII – 8 Bronchopneumonia, pneumonia abscedens

This group numbered 57 cases; 20 monoinfections and 37 polyinfections. Monoinfections were mostly caused by pyogenic *Staphylococci* (9 cases), *Pseudomonas* (8 cases) and *Klebsiella* bacilli (3 cases). In polyinfections predominating were: *Pseudomonas* (25 cases), *Klebsiella* and *Escherichia* (23 cases each) and pyogenic *Staphylococci* (22 cases), less frequent were cases with *Proteus* (7 cases). In 54 cases antibiotics administration proved to have no effect upon the course of infection. In 3 cases antibiotics were not applied. Phage therapy eliminated the suppurative process in 47 cases (82.5%) and in the remaining 10 cases it had unfavorable influence upon the infection. In 6 cases polyinfections resulted in exit.

Category VIII – 9 Pneumonia with thoracic empyema and fistulas

This group included 27 cases: 11 monoinfections and 16 polyinfections. The former ones were caused by pyogenic *Staphylococci* (6 cases), *Pseudomonas* (3 cases) and *Klebsiella* bacilli and *Escherichia* (1 case each). In polyinfections predominating were: *Pseudomonas* (13 cases), *Klebsiella* (10 cases), pyogenic *Staphylococci* (8 cases), *Escherichia* bacilli (8 cases) and *Proteus* (5 cases). Antibiotic treatment was ineffective in 23 cases. In 3 cases the treatment terminated only in a transient improvement and 1 in exit. In the remaining 23 cases (85.2%), suppression of the infection in the lungs and thoracic cavity was observed.

Category VIII – 10 Thoracic empyemas with fistulas

This groups numbered 22 cases of which 13 were monoinfections caused by pyogenic *Staphylococci* (6 cases), *Pseudomonas* (4 cases), *Klebsiella* (2 cases) and *Escherichia* (1 case), and 9 polyinfections caused by pyogenic *Staphylococci* (9 cases), *Pseudomonas* (7 cases), *Klebsiella* (5 cases) and *Escherichia* (4 cases). Antibiotic therapy was ineffective in 20 cases. In 2 cases antibiotics were not applied. Phage therapy eliminated the suppurative process in 19 cases (86.4%) and gave way to marked improvement in 2 cases. One case resulted in exitus.

Category IX – 11 Acute gingivitis, purulent peritonitis and abdominal abscesses

To this category 65 cases were included: 2 cases of acute purulent gingivitis and stomatitis and 63 cases of suppurative peritonitis due to suppurative or gangrenous acute appendicitis, gastric resection, cholecystectomy, suppurative pancreatitis and operations on intestines. Antibiotics proved ineffective in 63 cases. Thirty eight cases of monoinfections were mostly caused by *Escherichia* (20 cases), pyogenic *Staphylococci* (12 cases), *Pseudomonas* (3 cases) and *Klebsiella* (3 cases). Polyinfections were confirmed in 27 cases and were caused by *Klebsiella* (18 cases), *Pseudomonas* (17 cases) and *Escherichia* (17 cases), pyogenic *Staphylococci* (13 cases), and *Proteus* (4 cases). Bacteriophage were applied orally and locally (for the washing of operation field and to the fistulas); Good therapeutic result was reported in 59 cases (90.8%). In 5 cases marked improvement with the tendency toward heling of the wounds was noted and in 1 phage therapy was ineffective. Local application of bacteriophages in ulcerative gingivitis brought about full elimination of the suppurative process within 2 weeks.

Category X – 12 Suppurative inflammations of the genitourinary tract

This category comprised 42 cases: 40 cases of suppurative inflammation of the genitourinary tract and 2 cases of postoperative suppurative vaginitis. Monoinfections caused by *Pseudomonas* (8 cases), *Escherichia* (5 cases), pyogenic *Staphylococci* (3 cases), *Klebsiella* (1 case) and *Proteus* (1 case), were confirmed in 18 cases. Polyinfections numbered 24 cases and were caused by *Pseudomonas* (16 cases), *Escherichia* (16 cases) and pyogenic *Staphylococci* (14 cases), *Klebsiella* (12 cases) and *Proteus* bacteria (4 cases). In 37 cases antibiotic therapy was without effect. Application of phage therapy in 39 cases (92.9%) resulted in elimination of the infection and sterilization of urine. One patient revealed marked improvement and 2 – lack of improvement.

In 2 cases of suppurative vaginitis due to infection with pyogenic *Staphylococci*, the washings with the specific bacteriophages totally eliminated the inflammation.

Category XII – 13 Furunculosis

This groups included 55 cases of furunculosis of various localization. In 50 cases monoinfections caused by pyogenic *Staphylococci* were confirmed. In 5 cases of polyinfection, the presence of pyogenic *Staphylococci* (5 cases), *Escherichia* (3 cases) pyogenic *Staphylococci* and *Pseudomonas* (1 case each) was confirmed. All the cases were resistant to antibiotic treatment. Good therapeutic result was obtained in all the cases (100%) after oral and local application of bacteriophages.

Category XII — 14 Diseases of the connective tissue and lymphatic vessels

This group included 91 cases of skin inflammations: abscesses, phlegmone of various localization, acne necrotica and 11 cases of abscesses mammae. Seventy four cases appeared to be monoinfections caused by pyogenic *Staphylococci* (65 cases), *Pseudomonas* (5 cases), *Klebsiella* (3 cases), *Escherichia* (1 case). In 17 cases of polyinfections there were isolated pyogenic *Staphylococci* (15 cases), *Klebsiella* (10 cases), *Escherichia* (7 cases), *Pseudomonas* (9 cases) and *Proteus* (4 cases). In 83 cases antibiotic treatment appeared ineffective. Phage therapy eliminated the infection in 86 cases (94.5%). Five cases revealed marked improvement with tendency towards healing.

Category XII — 15 Decubitus ulcer

Of 16 cases of long-term decubitus ulcer, monoinfections were confirmed in 2. Pyogenic *Staphylococci* were recognized to be the causative factor. In 14 cases of polyinfection, different bacterial flora was encountered: pyogenic *Staphylococci* (11 cases), *Pseudomonas* (10 cases), *Escherichia* and *Klebsiella* (9 cases each) and *Proteus* (6 cases). All the cases were resistant to antibiotics. Application of phage therapy, oral and local, resultated in favorable effect in 13 cases. In 3 cases phage treatment was ineffective.

Category XIII — 16 Purulent arthritis and myositis

This group numbered 19 cases: 15 cases of purulent arthritis and 4 cases of myositis. Thirteen of them were monoinfections caused by pyogenic *Staphylococci* (12 cases), *Proteus* (1 case). Polyinfections numbering 6 cases were caused by pyogenic *Staphylococci* (5 cases), *Pseudomonas* (3 cases), *Proteus* (4 cases), *Klebsiella* (2 cases) and *Escherichia* (2 cases). All the cases were resistant to antibiotic treatment. Application of phage therapy eliminated the suppurative process in 17 cases (89.5%). In 2 cases no improvement was reported.

Category XIII — 17 Osteomyelitis of the long bones

This group included 40 cases of osteomyelitis of long bones. Monoinfections confirmed in 29 cases were due to infection with pyogenic Staphylococci (25 cases) in 3 cases *Pseudomonas* bacilli were isolated and in 1 *Klebsiella* bacilli. In 11 cases mixed infections was confirmed caused by pyogenic *Staphylococci* (7 cases), *Pseudomonas* (5 cases), *Proteus*, *Klebsiella* and *Escherichia* (4 cases each).

Thirty eight of 40 cases appeared resistant to antibiotics. Suppurative process was eliminated due to application of phages in 38 cases (95.0%). Two cases revealed a transient improvement.

Category XVII – 18 Open wounds, laparotomy, tracheostomy, contusions and burns

This group included 49 cases (22 monoinfections and 27 polyinfections). The former ones were caused by pyogenic *Staphylococci* (11 cases), *Pseudomonas* (5 cases), *Escherichia* (4 cases) and *Klebsiella* (2 cases). In polyinfections the presence of various bacterial flora was confirmed consisting maily of *Klebsiella* (16 cases), *Pseudomonas* (14 cases), *Escherichia* (16 cases), *Proteus* (9 cases) and pyogenic *Staphylococci* (15 cases). Antibiotic therapy was ineffective in 46 cases. Oral and local application of bacteriophages eliminated the infection in 42 cases and in 7 brought about a transient improvement.

Category XVII – 19 Contusions of head with suppuration of wounds

This group comprised 23 cases, including 13 cases of monoinfection and 10 of polyinfection. In monoinfections the suppuration was due mostly to pyogenic *Staphylococci* (10 cases), rarely *Klebsiella*, *Escherichia* and *Pseudomonas* (1 case each). In polyinfections frequently met were: *Pseudomonas* (9 cases), *Klebsiella* (7 cases), pyogenic *Staphylococci* (6 cases) and *Escherichia* (5 cases). The remaining 22 cases, with 1 exception, were resistant to antibiotics. Application of bacteriophages orally and locally, eliminated the infection in 19 cases (82.6%), in 2 resulted in marked improvement and in 4 in transient improvement.

Category XVII – 20 Injuries of spine (suppuration of wounds)

This group numbered 12 cases: 8 monoinfections and 4 polyinfections. In the former group pyogenic *Staphylococci* were predominating and in the latter they were encountered in 4 cases, *Klebsiella* and *Escherichia* bacilli in 2 cases each, and *Proteus* in 1 case. All of the cases were resistant to antibiotic treatment. Application of phage therapy allowed elimination of the suppurative process. Marked improvement with the healing of the wound and transient improvement were reported in 1 case each.

Category XVII – 21 Ostitis of the long bones after fracture

This group included 41 cases: 24 monoinfections and 17 polyinfections. Monoinfections were caused mainly by pyogenic *Staphylococci* (21 cases), *Escherichia* and *Proteus* (1 case each). In polyinfections predominating were pyogenic *Staphylococci* (14 cases), *Pseudomonas* (10 cases), *Klebsiella* (6 cases), *Escherichia* (7 cases) and *Proteus* (3 cases). Resistance to antibiotics was

confirmed in 37 cases. Bacteriophages applied orally and locally eliminated the infection in 37 cases (90.2%). In 4 cases only a transient improvement was reported.

Category XVII – 22 Chronic suppurative fistulas

This group comprised 180 cases in which the infections resulted in long-persisting suppurative fistulas. Monoinfections (118 cases) caused by pyogenic *Staphylococci* numbered 92 cases. *Escherichia* bacilli were recognized in 13 cases, *Pseudomonas* in 6 cases and *Klebsiella* in 7 cases. In polyinfections (62 cases) predominating were: pyogenic *Staphylococci* (38 cases), *Klebsiella* (33 cases), *Pseudomonas* (30 cases), *Escherichia* (31 cases) and *Proteus* (15 cases). In all the cases observed antibiotics were not effective. Bacteriophages applied orally and locally (to the fistulas) eliminated the infection in 168 cases (93.3%) and healed the fistulas. Marked improvement with the tendency toward healing of the wounds was reported in 12 cases.

RECAPITULATION OF THE RESULTS AND CONCLUSIONS

Detailed analysis of 550 cases of suppurative bacterial infections subjected to phage therapy allowed the following conclusions:

1. Specific phage therapy is highly effective in the infections caused by pyogenic *Staphylococci* and Gram-negative bacteria (*Klebsiella*, *Escherichia*, *Proteus*, *Pseudomonas*). Positive therapeutic result was observed in 508 cases of 550 examined, i.e. in 92.4%. This percentage ranged from 75.9% to 100%, in accordance with etiologic factor and type of infection.

2. Differences in positive results of phage therapy in the age group up to 60 years achieved statistical significance as compared to the age group above 60 years.

3. Statistically significant differences related to the sex of patients were also confirmed.

4. Difference in effectivity of phage therapy in monoinfections and polyinfections were statistically significant. They were most drastic in the group of infections caused by Gram-negative bacilli. In the *Staphylococcal* infections they were negligible.

5. Phage therapy is particularly useful in the cases where antibiotic therapy failed. In the material studied the number of cases resistant to antibiotics was as high as 518, i.e. 94.2%.

6. Phage therapy is recommended in the following pathological states:

a) acute infections of the alimentary tract (bacillary dysentery, diarrhoeas caused by *Salmonella*, *Klebsiella*, *Escherichia*, *Proteus* and *Pseudomonas*),

b) septicaemias irrespective of their origin,

c) postoperative infections irrespective of localization

d) suppurative skin diseases, diseases of the subcutaneous tissue, and

lymphatic vessels. Particular effectivenes should be underlinded in the treatment of furunculosis,

e) suppurative infections of the respiratory tract, lungs and pleura,

f) infections of the urinary tract,

g) suppurative infections of joints and bones,

h) suppurative fistulas.

7. Effectivity of phage therapy as compared to the combined treatment of phages and antibiotics revealed statistical significance.

8. Statistically significant differences related to the severity of the disease were also observed.

9. Bacteriophage treatment may last long, up to full elimination of the infective process. Side effects are rarely encountered.

10. The routs of bacteriophage administration depend upon a localization of suppuration focus. Oral administration of phages is recommended in each case. Bacteriophages easily penetrate from the alimentary tract to the blood circulation system and are excreted with the urine. With great effectiveness they were also applied as moist applications to the wounds, eye, ear and nose drops, infusions to the fistulas, washings of the nasal cavity, urinary bladder, suppurative lesions of pleura and peritoneum, decubitus, fistulas, intravaginally intraoperatively for the washing of peritoneal cavity and in the cases of multiple skin abscesses.

11. Bacteriophages are of great value in prophylaxis (in preparing the patients for orthopaedic surgeries, in skin transplantations).

12. Unfavorable treatment results may be accounted, to a great extend, to too late initiation of the treatment and also great cachexy of patients with long course of disease.

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Biodistribution of filamentous phage-Fab in nude mice

Yum L. Yip [a,b], Nicholas J. Hawkins [c], Glenn Smith [b], Robyn L. Ward [a,b,*]

[a] *School of Medicine (St. Vincent's Hospital), University of NSW, Sydney, NSW 2052, Australia*
[b] *CRC for Biopharmaceutical Research, Darlinghurst, NSW 2010, Australia*
[c] *School of Pathology, University of NSW, Sydney, NSW 2052, Australia*

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Abstract

In vivo panning of peptide libraries in mice has allowed the isolation of peptides which target the vasculature of specific organs. The application of this approach to phage displaying Fab fragments (phage-Fab) could lead to the isolation of antibodies which recognize novel tumor antigens. In this study, we have evaluated the biodistribution of phage-Fab in nude mice. Balb/c nude mice were injected intravenously with 10^9 TU of phage displaying the anti-colon cancer Fab c30.6. Blood samples were collected at nine time points over a period of 72 h and three groups of four mice were sacrificed at 4 min, 24 h and 72 h. Normal tissues (liver, colon, spleen, kidneys, lungs, skeletal muscle) and faeces were collected at these time points and the number of viable phage in each sample was determined. The distribution of phage in tissues was also examined by immunohistochemical analysis of paraffin-embedded tissues. Regression analysis of plasma kinetic data showed that the half-life and the volume of distribution of phage was 3.6 h and 1 ml, respectively. Phage uptake occurred predominantly in lungs, kidneys, spleen and liver. Relatively few phage were distributed to colon and muscle, and phage were eliminated from the circulation by 72 h. Immunohistochemical analysis showed phage to be mainly within the vasculature at 4 min, whereas notable phage extravasation was observed at 24 h and 72 h. In conclusion, this study provides information on the in vivo behavior of phage-Fab which will be useful in the design of in vivo panning strategies. By choosing appropriate time points for tissue collection, it may be possible to isolate novel Fabs against both intra- and extravascular targets.

Keywords: Biodistribution; Phage-Fab; Nude mice

1. Introduction

Antibody phage display has allowed the isolation of human antibodies recognizing a range of different antigens, including cell surface-expressed tumor antigens. Antibodies have been selected by panning phage libraries against purified antigens (Clark et al.,

Abbreviations: DAB, diaminobenzine tetrahydrochloride;; HRP, horseradish peroxidase; i.v., intravenous; PEG, polyethylene glycol; PMSF, phenylmethylsulphonylfluoride; TU, transducing unit

* Corresponding author. Department of Medical Oncology, St. Vincent's Hospital, Darlinghurst, NSW 2010, Australia. Tel.: +61-2-92958412; Fax: +61-2-92958451; E-mail: r.ward@garvan.unsw.edu.au

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1997; Desai et al., 1998) or against whole tumor cells (Cai and Garen, 1995; Pereira et al., 1997; Figini et al., 1998). The clinical application of these tumor-specific antibodies will depend on their ability to target tumor in vivo. Unfortunately, numerous studies have failed to demonstrate a correlation between the in vitro binding characteristics of antibodies and their in vivo tumor targeting properties (Matzku et al., 1987; Andrew et al., 1990; Boerman et al., 1990; Shockley et al., 1992b). Parameters such as antigen expression level and accessibility, as well as tumor physiology, have a profound influence on in vivo antibody targeting (Jain, 1990; Shockley et al., 1992a; Sung et al., 1992), yet these parameters cannot easily be predicted by in vitro studies.

Recently, peptides that selectively localize to the vasculature of various tissues have been isolated by the in vivo panning of random peptide libraries in mice (Pasqualini and Ruoslahti, 1996; Rajotte et al., 1998). This approach has considerable potential in the isolation of antibodies with good in vivo targeting properties. In particular, panning phage displayed Fab libraries in live animals may allow the identification of antibodies that recognize biologically relevant tumor targets, and may also facilitate the discovery of novel surface tumor antigens.

The successful application of in vivo panning to the isolation of tumor specific antibodies must inevitably be predicated on an understanding of the pharmacokinetics of phage-Fab. This includes a knowledge of the biodistribution of phage in different tissues, their clearance from the circulation, and their capacity for extravasation into the interstitial compartment. An understanding of the last point is of particular importance in those studies seeking to isolate Fab with reactivity against extravascular antigens. To gain a better understanding of these parameters, we have investigated the pattern of distribution of phage-Fab in nude mice.

2. Materials and methods

2.1. Balb/c nude mice

Six to eight week old female Balb/c nude mice were purchased from Animal Resource Centre (Canning Vale, Western Australia), and were held in micro-isolators. All experiments were conducted with the approval of the Institutional Animal Experimentation Ethics Committee.

2.2. Amplification of phage from single colony

The Fab region of the chimeric anti-colon cancer antibody c30.6 (Mount et al., 1994) was PCR amplified and cloned into the MCO1 phage display vector (Ward et al., 1996). The nucleotide sequence of the antibody was verified by dideoxy-sequencing. A single *E. coli* XL1-Blue colony harbouring the c30.6 clone was grown overnight at 37°C in 2YT supplemented with 50 μg/ml carbenicillin, 10 μg/ml tetracycline and 2% glucose (2YT/carb/tet/glu). The overnight culture (1 ml) was inoculated into 100 ml fresh 2YT/carb/tet/glu and grown for 3 h at 37°C with shaking. VCSM13 helper phage (5×10^{11}) were added and the culture was incubated for an additional 2 h at 37°C with shaking. Cells were pelleted at $1600 \times g$, resuspended in 100 ml fresh 2YT/carb/tet containing 70 μg/ml kanamycin and grown overnight with shaking at 30°C. The next day, cells were pelleted at $6000 \times g$ and the phage in the supernatant were precipitated at 4°C with 1/5 volume 20% PEG 6000/2.5 M NaCl. Phage were pelleted by centrifugation at $10{,}000 \times g$ and resuspended in PBS to a final concentration of 10^{14} TU/ml.

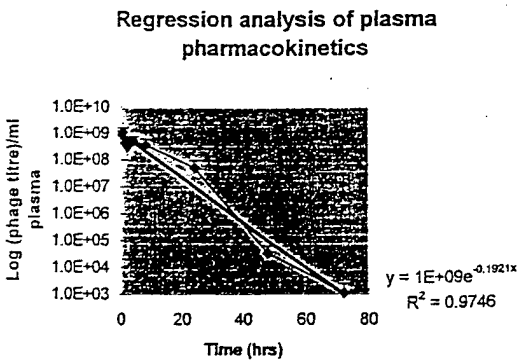

Fig. 1. Plasma pharmacokinetics of phage c30.6 in nude mice injected with 1×10^9 TU. The mean phage titre at each time point in three mice is shown together with S.E.

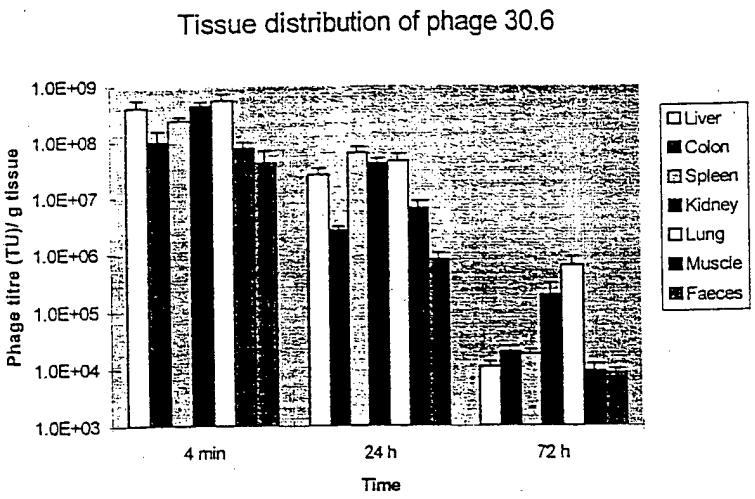

Fig. 2. Distribution of phage in tissues after 4 min, 24 h and 72 h following i.v. injection of 1×10^9 TU of phage c30.6. Values expressed as phage transducing unit (TU) per gram of tissue. Bars represent standard error (S.E.)

2.3. Calculation of plasma kinetic data

Three groups of three nude mice were injected i.v. with 1×10^9 c30.6 phage in a volume of 100 μl PBS. Blood samples were taken from the first group of mice at 4 min, 30 min, 1 h; from the second group of mice at 2 h, 4 h, 8 h; and from the third group of mice at 24 h, 48 h and 72 h. A 50 μl aliquot of plasma was incubated with 1 ml of log phase *E. coli* XL1-Blue for 30 min at 37°C without shaking. Following the addition of 10 ml of LB supplemented with 20 μg/ml carbenicillin, the cultures were incubated for another 1 h with shaking. Various dilutions of the culture were plated in duplicate on LB agar supplemented with 10 μg/ml carbenicillin and phage titres were determined by colony counting. Log percentage of initial injected phage was plotted against time, and assessed by regression analysis. The half-life ($t_{1/2}$) of the phage and the volume of distribution (V_d) were calculated using standard methods (Welling, 1986).

2.4. Quantitative distribution study of phage in mouse tissues

Three groups of four nude mice were injected i.v. with 1×10^9 c30.6 phage in a volume of 100 μl PBS. Mice were sacrificed at 4 min, 24 h and 72 h after injection. Faeces and normal tissues (liver, colon, spleen, kidneys, lungs, skeletal muscle) were weighed and assayed for the presence of infective phage. Tissues were ground into fine pieces in 1 ml of DMEM (Trace Biosciences, NSW, Australia) supplemented with 1 mM PMSF and protease inhibitor cocktail tablet (Boehringer Mannheim, NSW, Australia) (DMEM-PI). The mixture was clarified by centrifugation at $2000 \times g$ and the supernatant was kept for phage titering. The tissue pellets were washed three times in 3 ml of DMEM-PI supplemented with 0.1% BSA, and resuspended in 1 ml 0.1 M glycine pH 3.0 for 10 min at room temperature. This solution was neutralized with 1/10 volume of 1 M Tris pH 8.0 prior to centrifugation. Aliquots from

Table 1
Tissue to blood ratio of phage c30.6 at various time points

	Liver	Colon	Spleen	Kidneys	Lungs	Muscle	Faeces	Plasma
4 min	0.23	0.06	0.13	0.25	0.31	0.04	0.00	1
24 h	0.07	0.01	0.16	0.10	0.12	0.02	0.00	1
72 h	5.0	9.1	8.2	86.4	286	4.1	3.8	1

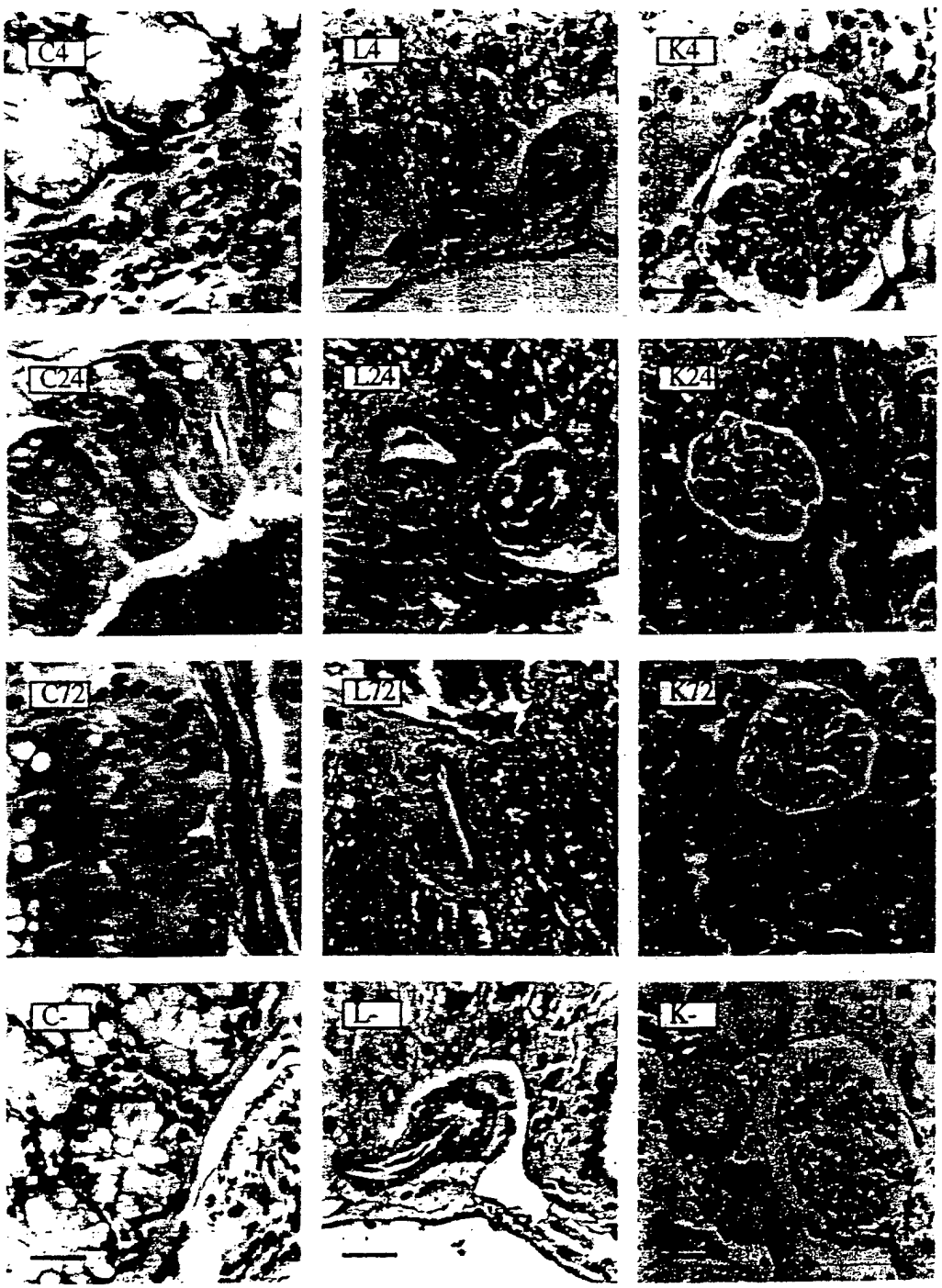

Fig. 3. Immunohistochemical staining of mouse tissue sections (C = colon, L = liver, K = kidney) after i.v. injection of phage c30.6 (4 = 4 min, 24 = 24 h, 72 = 72 h). Tissue sections were stained with a sheep anti-M13 antibody followed by a secondary HRP-conjugated donkey anti-sheep antibody. Negative controls (−) were stained with the HRP-conjugated donkey anti-sheep antibody only. Bar represents 50 μm.

the supernatant, washes and glycine elution were used to infect 1 ml of log phase XL1-Blue for 30 min at 37°C with no shaking, and phage titres determined as described above.

2.5. Immunohistochemical staining of mouse tissue sections

Paraffin-embedded tissue sections were prepared from mice injected with phage. The sections were dewaxed, rehydrated and endogenous peroxidase activity was blocked with 3% H_2O_2/H_2O for 5 min at room temperature. The sections were then blocked with normal rabbit serum for 1 h at 37°C, and incubated with 10 μg/ml sheep anti-M13 (Pharmacia Biotech, NSW, Australia) in 2% BSA/TBS. Following one wash in TBS for 5 min, donkey anti-sheep-HRP (2 μg/ml; Jackson ImmunoResearch, NSW, Australia) in 2% BSA/TBS was applied to the sections for 1 h at room temperature. Bound secondary antibody was detected by incubation with DAB substrate (Sigma, NSW, Australia), and sections were counterstained in Harris hematoxylin, dehydrated and mounted. All sections were assessed by two of the authors (NJH, YLY).

3. Results

3.1. Distribution of phage-Fab in plasma and tissue

The phage titres in the plasma at different time points were determined and log percentage of initial injected phage was plotted against time (Fig. 1). By using regression analysis it was determined that the half-life of phage in plasma was 3.6 h and the volume of distribution was 1.0 ml.

Titres of phage were determined in supernatant, washes and glycine elution of each tissue. At all time points, nearly all phage (90%) were recovered from the supernatant of ground tissues. Only 10% of the phage were found in the washes and 0.1% were isolated from the glycine elution. On this basis, supernatants were used in all subsequent assessments of the phage biodistribution (Fig. 2).

At 4 min and 24 h, phage were found predominantly in the liver, spleen, kidneys and lungs. Phage concentrations in colon, muscle and faeces were approximately one order of magnitude lower at those time points. At 72 h, the remaining phage had accumulated in the lungs and kidneys, and few phage ($< 10^4$ TU) were recovered from other tissues. In all tissues, the tissue:blood ratio of phage c30.6 was less than 1.0 at both 4 min and 24 h, indicating that phage were primarily found in the plasma at these times (Table 1). At 72 h, the tissue:blood ratio was greater than 1.0 in all tissues examined. Few phage were left in plasma suggesting that phage had been cleared from the circulation. At this time point, phage had accumulated mostly in the lungs (ratio = 286) and kidneys (ratio = 86).

3.2. Immunohistochemical analysis of phage distribution

Tissues collected at 4 min, 24 h and 72 h were embedded in paraffin, and 5 μm sections were stained with a sheep anti-M13 antibody. At 4 min, there was distinct staining of the lumenal surface of the capillary endothelium in all organs examined (Fig. 3). In particular, intense staining was found within the glomerular capillary loops at 4 min. No evidence of tissue uptake was found at this time point. At 24 h, however, phage were found to have left the vascular compartment, and were present in the parenchyma of liver, the red pulp of spleen, the pulmonary interstitium, the proximal convoluted tubules of kidneys and in faeces. No staining was found in the bowel wall or in skeletal muscle. At 72 h, the pattern of staining remained essentially unchanged from 24 h for all tissue sections, although the intensity of staining was reduced.

4. Discussion

In vivo panning represents a significant new application of phage display technology, with the panning steps moving from an in vitro system to a dynamic system dependent on circulation within a viable organism. The strategy involves the injection of phage into a mouse, with recovery from tissues followed by reamplification in vitro, and finally, the injection of amplified phage into a second mouse (Pasqualini and Ruoslahti, 1996). To fully realize the potential of this approach, a number of technical questions must be answered, and in particular, the

most appropriate time for tissue collection must be determined. Our study focused on the temporal distribution of phage-Fab in nude mice. These animals were chosen since they are routinely used in preclinical studies of antibody tumor targeting. The experiments were also performed with a monoclonal phage population (phage c30.6) known to have no specific activity against mouse tissues. Because of this, phage titres found in different organs could be expected to represent the background titres in the first round of an actual panning experiment, where only a tiny proportion of specific binding phage-Fab are present in the library.

The distribution of phage-Fab in normal tissues over time shows two main patterns. In one group of tissues, which includes liver, spleen, kidneys and lungs, the uptake of phage at 4 min and at 24 h was significantly greater than that seen in colon and skeletal muscle (Fig. 2). At 4 min, this difference could in part be due to the higher perfusion and capillary permeability of the liver, lungs and kidneys when compared with colon and skeletal muscle (Covell et al., 1986). At 24 h, the number of phage recovered from each organ was decreased by 10-fold but the pattern of distribution remained essentially unchanged, with a slightly lower tissue:blood ratio than at 4 min (Fig. 2 and Table 1). This suggests that while phage were nearly all distributed by 4 min, the majority of phage-Fab was still in the tissue blood volume during the first 24 h. Following distribution of phage, it is expected that normal blood flow would wash away non-specific or low affinity phage, thus mimicking the washing steps of in vitro panning. At 72 h, phage were eliminated from the circulation, and thus those remaining in the tissues represented extravasated and trapped phage. The distribution of phage was also influenced by the normal structure and function of individual organs. Not unexpectedly, high phage uptake was found in the liver, lungs and spleen because of uptake into the reticulo-endothelium system, a finding also reported by Pasqualini et al. (1997).

A further factor which clearly influenced the tissue distribution of phage-Fab was their size. The filamentous phage M13 are shaped like flexible filaments with a diameter of 65 Å and a length of 9300 Å (Glucksman et al., 1992), whilst the dimension of the Fab they display is 80 Å × 50 Å × 40 Å (Poljak et al., 1973). As such, it is likely that the extravasation of phage would be restricted by the continuous endothelium of capillaries in tissues such as skeletal muscle, skin, connective tissues and brain (Clough, 1991). However, in organs such as liver, spleen, lungs and kidneys, immunohistochemical staining demonstrated that phage extravasation occurred by 24 h. The vasculature of the liver and spleen are discontinuous and contain open fenestrae (600 Å in diameter) backed by a discontinuous basement membrane (Clough, 1991). The extravasation of phage in these tissues is likely to occur through this route. Thus the ability of phage to extravasate is related not only to the circulation time, but also to the ultrastructure of the vasculature in different tissues. Given the heterogeneous and leaky nature of the tumor vasculature (Dvorak et al., 1988), it is possible that by 24 h, phage may extravasate into the tumor interstitium, although high tumor interstitial pressure may reduce the penetration of phage-Fab (Jain, 1990).

Our study suggests that in nude mice, phage are largely eliminated by both hepatic and renal excretion. The phage are probably filtered through the glomeruli (strongly stained at 4 min) and enter the proximal convoluted tubule by 24 h. While there was strong immunohistochemical staining of faeces by 24 h, the phage titres were low at this time point. This suggests that the majority of staining in faeces was due to the presence of phage protein fragments, probably following elimination in bile. Immunohistochemical detection of phage clearly does not necessarily indicate the presence of intact phage. However, with the exception of faeces, positive immunohistochemical staining in tissues was found to correlate closely with phage infectivity.

Since the c30.6 Fab displayed on the surface of the phage does not bind to normal mouse tissues (Mount et al., 1994), the biodistribution pattern we found in this study should be applicable to any phage-Fab population. This was verified by conducting similar experiments with phage displaying Fab reactive with tetanus toxoid and with Fabs against human carcinoembryonic antigen which are known to be non reactive with mouse tissue. In both cases, a similar distribution pattern was observed (results not shown).

In conclusion, our study provides basic pharmacokinetic data relevant to the design of in vivo

panning experiments. It is clear that the timing of phage retrieval will be influenced both by the organ being examined and the specific location of the target antigen in relation to the microvasculature. While 4 min appears to be the best time point for the isolation of Fab against intravascular antigens, the 24 h or the 72 h time points may prove superior for the isolation of antibodies against extravascular antigens. A disadvantage of the 72 h time point is the low phage titre which may be insufficient for subsequent panning rounds. The future studies will therefore involve the panning of an antibody library in tumor-bearing nude mice using 24 h as a time point for tissue collection.

Acknowledgements

The authors wish to thank A/Prof. Ken Williams, St Vincent's Hospital, and Dr. Andrew McLachlan, University of Sydney, for valuable discussions regarding the pharmacokinetics of phage in mice. Yum L Yip is the recipient of the Australian Postgraduate Award.

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UNIVERSITY OF WASHINGTON SCHOOL OF MEDICINE

Division of Infectious Diseases, Immunology/Rheumatology
(206) 543-3207; FAX (206) 543-3184

Department of Pediatrics

1959 N.E. Pacific St.
Box 356320
Seattle, WA 98195-6320

September 22, 1998

David Krag and Lyn Oligino
Room E309A, Given Building
The University of Vermont
Burlington, Vermont 05405

Dear David and Lyn,

This letter is to confirm our collaboration on your project on the *in vivo* screening of phage-displayed random peptide libraries for small ligands which specifically bind tumors. There are two aspects of this project for which my expertise may prove especially useful. First, our laboratory has had over 30 years of experience injecting *E. coli* bacteriophage (ϕX174) intravenously into humans for the purpose of measuring antibody responses, under an IND (BB-IND-714) from the FDA. I will be happy to advise you on the proper preparation of phage for safe injection into humans and any other aspects of the use of phage clinically. In addition, because obtaining an IND from the FDA can be a long and arduous process, it will be worthwhile for us to explore the use of ϕX174 phage as a vehicle for the display of random peptide libraries, as the longtime use of bacteriophage ϕX174, given intravenously to almost 3000 humans, has been determined to be extremely safe, and, as mentioned, the use of bacteriophage ϕX174 in humans has already been approved by the FDA. I will be happy to provide you with ϕX174 bacteriophage and its optimal E. coli host strain for the construction of novel phage-displayed random peptide libraries in the ϕX174 strain. Such libraries will almost certainly be non-toxic for human screening experiments, although more conventional phage-displayed libraries in M13 strains of bacteriophage may also be safe.

I look forward to our collaboration on this exciting and extremely innovative proposal.

Sincerely,

Hans D. Ochs, M. D.
Professor, Department of Pediatrics

BACTERIOPHAGE ϕX174
PRODUCT INFORMATION
NEW DRUG LIMITED BY FEDERAL LAW TO INVESTIGATIONAL USE ONLY
BB-IND 714
Lot 2-96 (April 1996)

Origin of Material: The original sample of this bacteriophage ϕX174 preparation and the carrier strain of *E. coli* for propagation of the bacteriophage was obtained in 1964 from Professor Neal Groman, Department of Microbiology, University of Washington, who obtained it in 1962 from Professor Robert Sinsheimer, California Institute of Technology (1). Following passage and partial purification in our laboratory, aliquots were dispensed in 13 x 7 mm sterile plastic tubes, frozen and stored at -70°C. Products from this passage have been thawed, propagated and used in a variety of studies on numerous occasions. The bacteriophage has bred true with consistent patterns of replication, plaque formation, and has shown inhibition by antisera prepared against bacteriophage ϕX174 (1-7) in mice, rats, guinea pigs, rabbits, dogs, cattle, horses, macaques, baboons, and humans (8-73).

Details of Manufacture: In 1973 a tube of the 1964 phage was thawed and passaged three times to assess viability and to increase titer. Aliquots of this material, kept at -70°C, were thawed in December 1975, propagated and re-frozen (-70°C). This material was used in August 1977 to prepare Lot 8-77. For Lot 2-96, a vial of Lot 8-77 was passaged once to increase titer, then this material was used to prepare three separate large batches of bacteriophage by the standard propagation technique. The three batches were pooled following the ammonium sulfate precipitation step, and further purified by DEAE-cellulose chromatography using stepwise elution with ammonium acetate. The fractions containing maximum bacteriophage (from 0.06 to 0.08M) were combined and diluted with 0.1M ammonium acetate to give a final bacteriophage concentration of approximately 10^{11} pfu per ml. This material was passaged twice under sterile conditions through 0.22 μm membrane filters (Falcon) and placed in rubber-capped sterile vials. Final volume of the batch was 2803 ml; 501 vials containing 0.8 ml, 999 vials containing 1.8 ml, and 76 vials containing 2.8 ml were prepared. All were then frozen and stored at -70°C.

Safety Tests: Batch 2-96 was assayed by standard techniques and contained 1×10^{11} pfu/ml. A total of forty vials were taken at random from the initial, middle, and final stages of bottling; 20 were tested for sterility using fluid thioglycolate medium and incubation at 30°C, and 20 were tested for sterility using fluid soybean-casein digest medium and incubated at 20-22°C for 14 days. Positive controls were included to demonstrate growth promoting activity of the media used. (see FDA regulations, section 610-12).

Batch 2-96 was tested for pyrogens in three rabbits. Temperatures were recorded by a rectal thermocouple. Following temperature stabilization after immobilization in the holding unit, 0.6 ml (0.2 ml/kg), were rapidly injected i.v. through an ear vein. Temperatures were monitored for three hours. There was no significant increase in temperature (maximum rise 0.56°C; average temperature rise of all three rabbits 0.38°C). We elected to inject only ten times the dose used in humans because we felt it important to inject the material rapidly intravenously, as we do in humans, and not to cause harm to the rabbit by injecting the full 3 ml per kilogram i.v. as requested by FDA regulations. Furthermore, we felt that, should the material be contaminated, a dose ten times that used in humans would show an endotoxin reaction in rabbits, known to be very sensitive to endotoxin by a rise in temperature.

Toxicity Tests: Two guinea pigs were injected with batch 2-96 intraperitoneally with 5 ml (15ml/kg) (representing 750 times the human dose) and two mice were injected with 0.5 ml (25 ml/kg) intraperitoneally (representing 1250 times the human dose). The animals were carefully observed for 7 days. No ill effects of any kind were observed. All animals survived. None lost weight.

Antigenicity: The antigenicity of the material was assessed in two guinea pigs and three rabbits. Prompt primary antibody responses were observed on day 7 and day 14 after immunization; the guinea pigs showed a mean peak titer of 103 (K_v) and the rabbits a mean peak titer of 106 (K_v) comparable to titers of previous preparations, and to those reported in the literature. Lot 2-96 was given to six volunteers (three males, three females) and neutralizing antibody titers (Kv) measured following 1° and 2° immunizations. All six controls showed a normal antibody response demonstrating amplification, immunologic memory and IgM to IgG switch (Tables 1, 2) (Figures 1a, 1b, 1c, 2).

Final Product Composition: The bacteriophage as bottled is suspended in 0.10 M ammonium acetate with 0.001 M CaCl2. Final pH is pH 8.0. The concentration of bacteriophage is 10^{11} pfu/ml. The final protein concentration is 0.09 mg/ml, DNA concentration is 2.48 µg/ml, comparable to previous lots.

Label: Each vial has the following label attached firmly to its outer surface:

> Caution: New drug limited by Federal law to investigational use.
> Bacteriophage ϕX174, 1 x 10^{11} PFU/ml, Lot 2-96
> Hans D. Ochs, University of Washington, Seattle, WA 98195 1.8 (0.8) (2.8) ml

Proposed Use of Product: The WHO Committee on Primary Immunodeficiency diseases identified bacteriophage ϕX174 as a standard antigen for the assessment of the immune response in humans. We make this method of assessment available to other investigators both within the United States and overseas. For this purpose we supply this preparation, on request only, to responsible investigators for the study of identified, appropriate patients with primary or secondary immunodeficiency diseases, to assess in a standardized manner their humoral immune response.

Transfer of bacteriophage ϕX174 to other investigators: Since bacteriophage ϕX174 antigen is classified as an Investigational New Drug by the FDA, before requesting the material from our laboratory each investigator must have filed and obtained an IND for the use of bacteriophage ϕX174. For safety and product information, the IND holder may refer to BB-IND 714 provided to Dr. Hans Ochs, University of Washington, Seattle, WA. The bacteriophage will then be shipped to the investigators and administered under the supervision of that investigator to the patient(s).

The bacteriophage ϕX174 antigen is administered intravenously according to a standard protocol (*vide infra*). Serum samples will be collected at stated intervals and returned to our laboratory for assay accompanied by a completed patient information form describing the disease, the date and amount of phage given and reactions (if any) observed; a form indicating the dates of blood samples. In accordance with FDA requirements, alarming adverse reactions must be reported to us immediately by telephone, fax, or registered letter. Serum samples are assayed in our laboratory for antigen clearance and antibody formation, and the results are reported to the investigator.

Risks: It is generally accepted that bacteriophage ϕX174 is highly selective in its ability to attach, penetrate and replicate in the host strain, *E. coli C*. We have been unable to find documentation in the literature of bacteriophage producing cytopathic events in human cells. Approximately ten years ago, we had started a collaborative study with Milstein, Walker and Petricciani exploring the possibility of replication of bacteriophage ϕX174 DNA in nonhuman primates, using hybridization with a labeled phage DNA probe. In one of two monkeys, hybridizable ϕX174 DNA sequences were transiently associated with lymphocytes, but not granulocyte DNA; in this one instance, the sequences were present five days after bacteriophage inoculation, but were no longer detectable seven months later. The results suggest that while replication of ϕX174 DNA may occur in primates *in vivo*, the bacteriophage DNA is apparently edited from the host cells.

Adverse Reactions: In reviewing the literature, dating back more than 30 years, no side effects in human volunteers or patients were reported, neither by other investigators who immunized 76 patients and controls (68-73), nor by investigators using bacteriophage ϕX174 produced in our laboratory (27-67). Excepting the episodes of reactions to Lot 1-88, described below, we have not observed or heard of side effects following immunizations of over 1500 individuals with bacteriophage ϕX174.

In 1988 we produced a new lot (1-88), which was obtained by pooling three batches, each with a relatively low yield of infective particles. This lot was given in 1989 to four volunteers, one of whom reported the onset of "flu-like symptoms", including malaise, chills, and joint and muscle aches, four and one-half hours after a primary phage injection; these symptoms responded to Ibuprofen and resolved completely within 24 hours after immunization. One of the remaining volunteers, after being contacted, reported shortness of breath during the night following primary phage immunization; these symptoms spontaneously resolved the next morning. Subsequently, after contacting all co-investigators who had received Lot 1-88, we identified two patients who recalled symptoms following injection with Lot 1-88. A 35 year old patient with common variable immune deficiency had a very high bacteriophage neutralizing antibody response, limited to IgM, following primary and secondary immunization in 1988 (Lot 1-83); he was immunized a third time, in 1989, to determine if he had developed lasting immunologic memory limited to the production of phage-specific antibody of the isotype after multiple exposure to the antigen. A few hours after receiving Lot 1-88, the patient developed fever, fatigue and arthralgia/myalgia; he was treated by his physician with aspirin and bed rest until disappearance of symptoms 4-6 days after immunization. The patient completely recovered without any sequelae. A second patient, a 10 year old boy with immunodeficiency and atopic symptoms, developed wheezing and shortness of breath seven minutes after his second immunization with Lot 1-88, requiring epinephrine injection and treatment with a nebulizer. He recovered completely within 30 minutes after immunization. Lot 1-88 was recalled by our laboratory. Subsequent lots (7-89; 10-92), used during the last five years, were not associated with reactions.

Review of the literature and our own data indicate that adverse events following immunization with bacteriophage ϕ X174 are minor and of low frequency. However, risks as yet unrecognized may exist, and patients receiving this material should be informed of such possibilities prior to consenting to this procedure.

Immunization protocol: The original protocol for phage immunization was designed in 1972 (27, 32) This protocol has been included in a report by the WHO Committee on Primary Immunodeficiency Diseases, which identifies bacteriophage ϕX174 as a standard antigen for the assessment of the immune response in man. Effective March 4, 1994, bacteriophage ϕX174 will be made available to investigators outside the state of Washington only if the investigator has obtained an IND from the FDA to use bacteriophage ϕX174 in humans. FDA-approved investigators with documentation will receive bacteriophage ϕX174 of a batch approved by the FDA for use of assessing antibody responses in man. In addition, investigators may request assistance in setting up the neutralization and ELISA techniques in their own laboratories. If the investigator prefers, we will assay each sample for the presence of phage-specific antibody by neutralization or by ELISA.

Bacteriophage ϕX174 is given intravenously at a dose of 0.02 ml/kg body weight using a standard preparation with 1×10^{11} infective phage particles per ml. (27, 32). Blood is obtained before immunization and 15 minutes post primary immunization. Subsequently, a 7 ml aliquot of blood is withdrawn at one week, two weeks, four weeks and six weeks post primary immunization. A secondary immunization is given at six weeks at a similar dose based on body weight. Blood is drawn at one, two and four weeks post secondary immunization. Each serum sample is assayed for antibody by neutralization or by ELISA (58). The one week post primary immunization is routinely checked for the presence of circulating bacteriophage. Phage-specific antibody of the IgG class is determined either by treating the sample with 2 mercaptoethanol (neutralization) (27, 32) or by using specific antisera to differentiate phage-specific IgG and IgM antibody by ELISA (58).

Those patients with an abnormal secondary response consisting of a low proportion of IgG may be selected for a tertiary immunization with bacteriophage to determine if the patient in fact is not capable of switching from IgM to IgG (58) and if the total antibody titer is less than the geometric mean of normals (to avoid potential adverse effects as described under "Adverse Reactions"). This will have therapeutic implications.

In patients with a "transient" immune deficiency following bone marrow transplantation or chemotherapy, it may be necessary to check the specific antibody response to phage once a year to determine when replacement therapy with IVIG is no longer necessary.

Bibliographic Information: The use of bacteriophage ϕX174 as an antigen has been widely studied in both animals and humans. Review of the literature and personal experience indicates that bacteriophage is a potent antigen. Because there is no natural exposure to this antigen, a consistent primary response on first immunization is observed in immunologically normal individuals. Following a secondary immunization, the antibody response shows amplification and switch from IgM to IgG; approximately 50% of the antibody is of the IgG isotype. Subsequent exposures to the antigen results in a "tertiary" response, showing almost exclusively phage-specific antibody of the IgG class (IgG over 90%) (Table 2). Furthermore, since neither humans nor animals are exposed to this antigen by natural means, no pre-existing antibodies exist and immunoglobulin preparations produced commercially for treatment do not contain phage-neutralizing antibody. Therefore, bacteriophage ϕX174 can be used to measure antibody responses in patients with primary immunodeficiency receiving IVIG infusions. Bacteriophage is particularly useful in identifying patients with X-linked agammaglobulinemia, a majority of whom lack B cells and are unable to mount an antigen-specific antibody; most of these patients are unable to clear bacteriophage ϕX174 immunologically (27, 32). Patients with functional B cells but lack of T help show a characteristic response to bacteriophage characterized by lack of amplification and failure to switch from IgM to IgG (27, 29, 32, 37, 38, 41, 52, 62, 64). Since the assay to assess neutralizing antibody is extremely sensitive, it is possible to detect minute amounts of antibody in patients who produce less than 0.01% of normal antibody responses. Thus, based on the experience obtained by our group and by others, bacteriophage induces a characteristic primary, secondary and tertiary antibody response in immunologically normal controls and characteristic patterns of responses in patients with primary or secondary immune deficiency disorders.

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Table 1

Normal Controls Immunized with Bacteriophage ϕX174, Lot 2-96

1° response to bacteriophage ϕX174

control	sex	1 week	2 weeks	4 weeks	6 weeks (pre 2 °)
RB	M	21.1	898	427	441
MB	F	2.5	227	140	59
KG	F	2.1	37	60	36.5
CG	F	5.6	312	99	51
JL	M	5.6	379	156	86
KO	M	3.75	202	220	109

2° response to bacteriophage ϕX174

control	sex	1 week	2 weeks	4 weeks	6 weeks (pre 2 °)
RB	M	1199	600	311	34%
MB	F	2544	1848	880	50%
KG	F	568	518	192	44%
CG	F	1049	436	173	64%
JL	M	1808	733	313	28%
KO	M	1006	825	363	42%

Table 2

Immunization with Bacteriophage ϕX174
Neutralizing Antibody (Kv), Normal Controls

	1°				2°					3°			
	1 week	2 weeks	4 weeks	6 weeks (pre 2°)	1 week	2 weeks	4 weeks	% IgG	pre 3°	1 week	2 weeks	4 weeks	% Ig
males (n = 12)									n = 5				
geometric mean	11.3	50.7	35.6	15.9	362	258	105	45%	22	1227	857	506	87%
66% confidence	4.4-28.9	13-202	12.3-103	6.3-40.3	207-631	161-414	56-196	28-71	2.7-175	1002-1502	697-1056	299-858	74-10
females (n = 19)									n = 9				
geometric mean	11.0	180	78	34	521	338	183	52%	161	901	863	1117	90%
66% confidence	5.2-23	66-496	31-195	13-88	333-816	202-567	108-311	38-70	37-697	390-2078	430-1733	480-2599	76-10
males + females (n = 33)									n = 14				
geometric mean	10.8	94	48	21	396	283	145	46%	85	990	861	842	89%
66% confidence	4.8-25	21-412	13.6-168	6.3-72	171-915	153-522	77-271	28-74	14-504	489-2006	499-1486	369-1920	75-10

FIGURE 1a

IMMUNE RESPONSE TO BACTERIOPHAGE PHI-X 174

Hans D. Ochs, M.D.
Department of Pediatrics RD-20
School of Medicine
University of Washington
Seattle, WA 98195
(206) 543-3207

Date: May 2, 1994

Patient's Name: Normal Control

Hospital Number: 0000

Investigator: Hans Ochs, M.D.

Institution: University of Washington

Address: Department of Pediatrics

Date Primary Given: 2-15-94

Date Secondary Given: 3-29-94

SUMMARY OF RESPONSE

Clearance: Yes

Primary: Normal

Secondary: Normal

	Log 1000 x O.D. NORMAL VALUES			PATIENT
	G.M.*	-2S.D.	+2S.D.	
Pri-1	2.059	1.606	2.512	2.357
Pri-2	2.475	1.973	2.977	2.745
Pri-4	2.530	2.130	2.931	2.761
Pre-2	2.585	2.101	3.069	2.910
Sec-1	3.143	2.955	3.331	3.214
Sec-2	3.140	2.982	3.298	3.220
Sec-4	3.066	2.876	3.257	3.157

Amplification (Memory): Yes

Immunoglobulin Class of Secondary: 47 %IgM

53 %IgG (normal, 53 +/- 32)

*Geometric Mean

FIGURE 1b

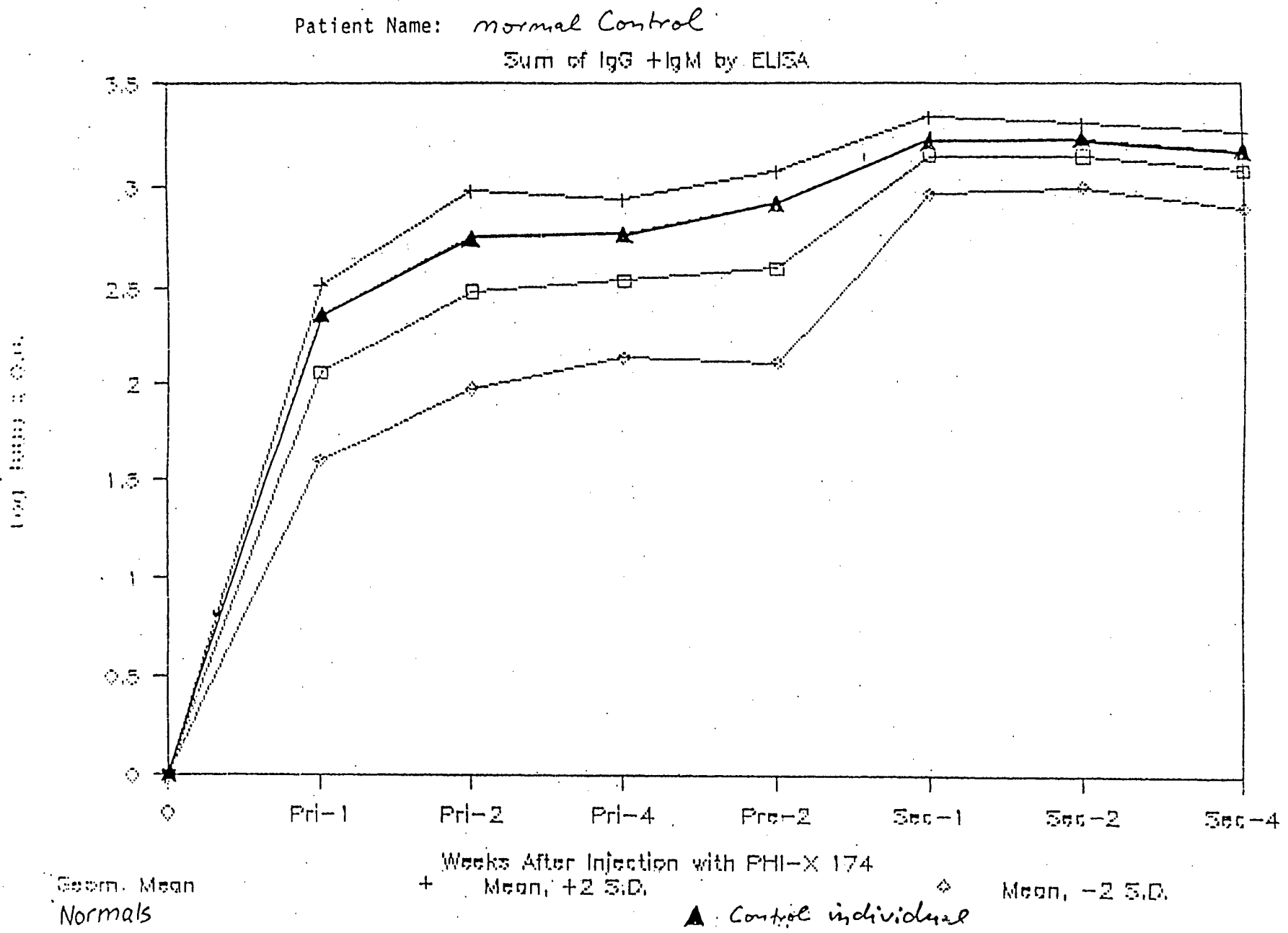

Patient Name: normal Control
Sum of IgG + IgM by ELISA
3.5
3
2.5
2
1.5
1
0.5
0
Pri-1
Pri-2
Pri-4
Pre-2
Sec-1
Sec-2
Sec-4
Weeks After Injection with PHI-X 174
Geom. Mean
Normals
Mean, +2 S.D.
Mean, -2 S.D.
Control individual

FIGURE 1c

Patient Name: Normal Control

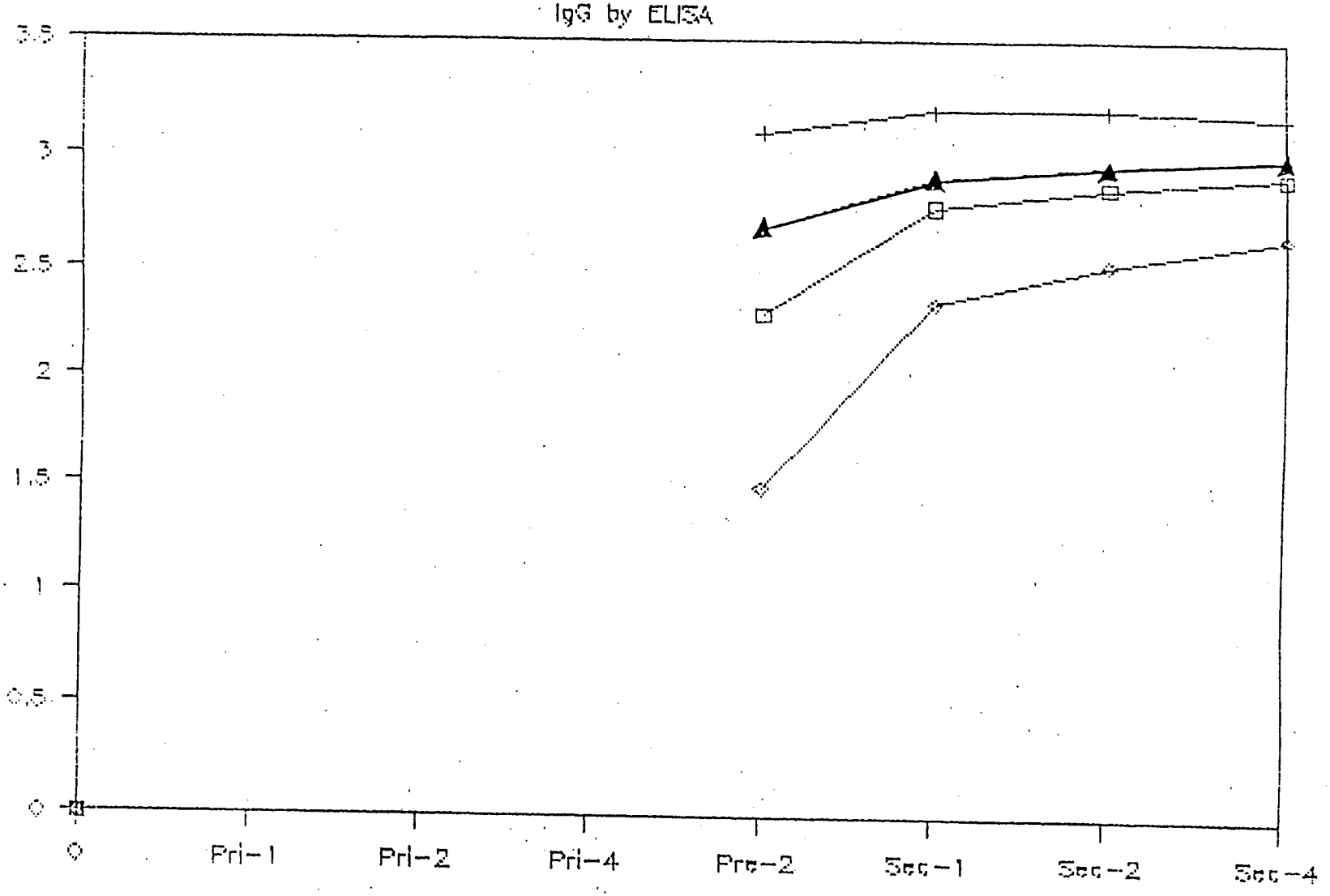
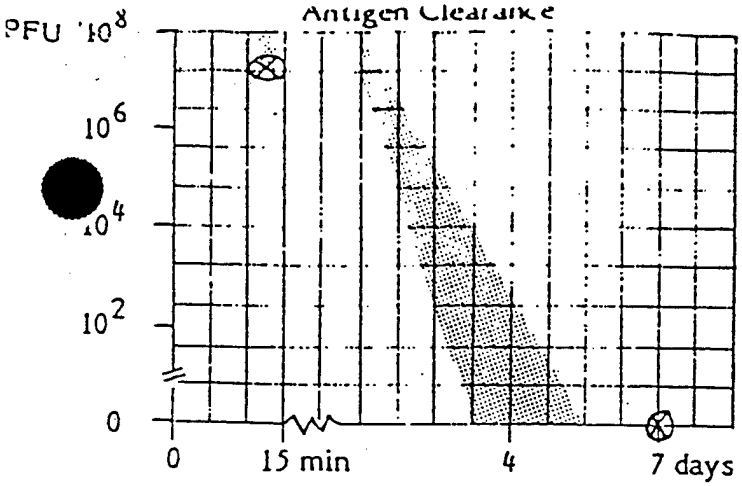
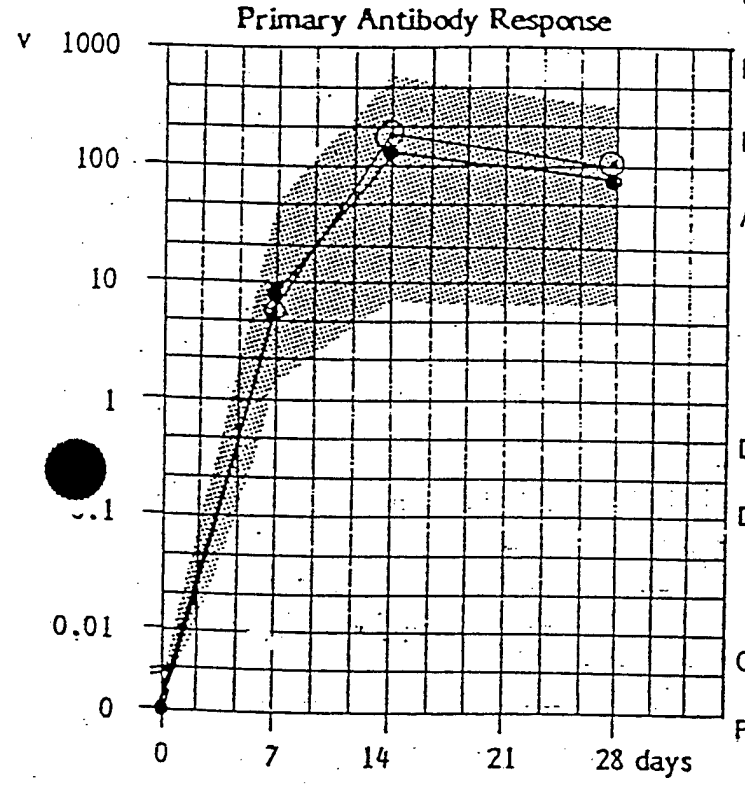


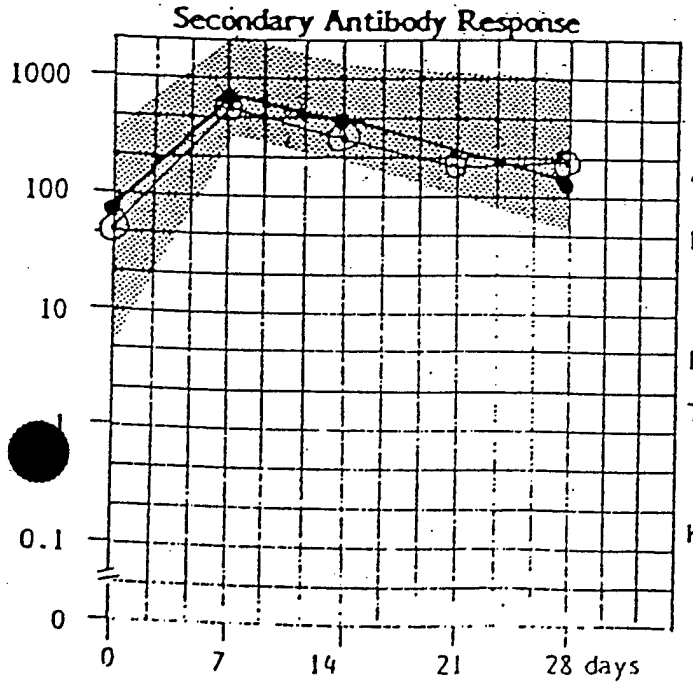
FIGURE 2

IMMUNE RESPONSE TO BACTERIOPHAGE ϕX174



Hans D. Ochs, M.D.
Department of Pediatrics, RD-20
School of Medicine
University of Washington
Seattle, WA 98195
(206) 543-3207





DATE _____

Patient's Name: Normal Control

Hospital Number: 0000

Investigator: Hans D. Ochs, M.D.

Institution: Department of Pediatrics

Address: UW School of Medicine

1959 NE Pacific

Seattle, WA 98195

Date Primary Given 2/15/94

Date Secondary Given 3/29/94

Summary of Response

Clearance: Yes

Primary: Normal

Peak Kv: 203.

Secondary: Normal

Peak Kv: 600.

Amplification (Memory): Yes

Ig class of secondary:

43 % IgM 57 % IgG (normal: 56 ± 24)

Response Classification:

Type: Normal ϕX control

Lot 10-92

Key:

▨ Normal range

O—O Patient (1 normal control) MdY

● --● Geometric mean of normal controls

BACTERIOPHAGE ϕX174
A T-DEPENDENT ANTIGEN
TO MEASURE ANTIBODY RESPONSES IN VIVO

INFORMATION FOR INVESTIGATORS

1. Use of bacteriophage ϕX174 to assay antibody responses in man and animals:

Bacteriophage ϕX174 has been used since 1962 to measure antibody responses in nonmammalian vertebrates (9), in mammalian vertebrates (8, 10-26) and in man (27-73). The data published clearly indicate that bacteriophage ϕX174 is a T cell dependent neoantigen (23, 32, 51, 52). Following a primary immunization, a typical IgM response is listed. Following secondary immunization, mammalian response is characterized by amplification of the antibody titer and switch from IgM to IgG; in man, approximately 50% of this antibody is IgG. Following a tertiary immunization, the antibody response is predominantly IgG (over 90%). Animals with congenital primary immunodeficiency diseases (15, 17-20, 22, 25, 26) and humans with congenital immune deficiency (27, 29, 31, 32, 37-41, 45, 47, 53, 59, 62, 63, 65-67, 69) have clearly abnormal responses to bacteriophage. Similarly, secondarily immunosuppressed animals (12, 13, 14, 16, 23) and humans (28, 30, 33-36, 42-44, 49, 50, 52, 57, 61, 68) have abnormal antibody responses that are useful to assess the severity of the immune suppression and, under certain circumstances, to follow the progress of immune reconstitution (12, 28, 30, 33, 35, 42-45, 50, 54, 61, 62).

Proposed Use of Product: The WHO Committee on Primary Immunodeficiency diseases identified bacteriophage ϕX174 as a standard antigen for the assessment of the immune response in humans. We make this method of assessment available to other investigators both within the United States and overseas. For this purpose we supply this preparation, on request only, to responsible investigators for the study of identified, appropriate patients with primary or secondary immunodeficiency diseases, to assess in a standardized manner their humoral immune response.

2. Description of product:

The original sample of bacteriophage ϕX174 currently used in our laboratory and the carrier strain of *E. coli* for propagation of the bacteriophage was obtained in 1964 from Professor Neil Groman, Department of Microbiology, University of Washington, who obtained both in 1962 from Professor Robert Sinsheimer, California Institute of Technology (1). This material has been passaged and partially purified in our laboratory and has been stored in aliquots at -70°C. From this stock phage, numerous lots of bacteriophage were prepared and used in immunizing animals and humans.

Details of Manufacture: In 1973 a tube of the 1964 phage was thawed and passaged three times to assess viability and to increase titer. Aliquots of this material, kept at -70°C, were thawed in December 1975, propagated and re-frozen (-70°C). This material was used in August 1977 to prepare Lot 8-77. For Lot 2-96, a vial of Lot 8-77 was passaged once to increase titer, then this material was used to prepare three separate large batches of bacteriophage by the standard propagation technique. The three batches were pooled following the ammonium sulfate precipitation step, and further purified by DEAE-cellulose chromatography using stepwise elution with ammonium acetate. The fractions containing maximum bacteriophage (from 0.06 to 0.08M) were combined and diluted with 0.1M ammonium acetate to give a final bacteriophage concentration of approximately 10^{11} pfu per ml. This material was passaged twice under sterile conditions through 0.22 μm membrane filters (Falcon) and placed in rubber-capped sterile vials. Final volume of the batch was 2803 ml; 501 vials containing 0.8 ml, 999 vials containing 1.8 ml, and 76 vials containing 2.8 ml were prepared. All were then frozen and stored at -70°C.

Safety Tests: Batch 2-96 was assayed by standard techniques and contained 1 x 10^{11} pfu/ml. A total of forty vials were taken at random from the initial, middle, and final stages of bottling; 20 were tested for sterility using fluid thioglycolate medium and incubation at 30°C, and 20 were tested for sterility using fluid soybean-casein digest medium and incubated at 20-22°C for 14 days. Positive controls were included to demonstrate growth promoting activity of the media used. (see FDA regulations, section 610-12).

Batch 2-96 was tested for pyrogens in three rabbits. Temperatures were recorded by a rectal thermocouple. Following temperature stabilization after immobilization in the holding unit, 0.6 ml (0.2 ml/kg), were rapidly injected i.v. through an ear vein. Temperatures were monitored for three hours. There was no significant increase in temperature (maximum rise 0.56°C; average temperature rise of all three rabbits 0.38°C). We elected to inject only ten times the dose used in humans because we felt it important to inject the material rapidly intravenously, as we do in humans, and not to cause harm to the rabbit by injecting the full 3 ml per kilogram i.v. as requested by FDA regulations. Furthermore, we felt that, should the material be contaminated, a dose ten times that used in humans would show an endotoxin reaction in rabbits, known to be very sensitive to endotoxin by a rise in temperature.

Toxicity Tests: Two guinea pigs were injected with batch 2-96 intraperitoneally with 5 ml (15ml/kg) (representing 750 times the human dose) and two mice were injected with 0.5 ml (25 ml/kg) intraperitoneally (representing 1250 times the human dose). The animals were carefully observed for 7 days. No ill effects of any kind were observed. All animals survived. None lost weight.

Antigenicity: The antigenicity of the material was assessed in two guinea pigs and three rabbits. Prompt primary antibody responses were observed on day 7 and day 14 after immunization; the guinea pigs showed a mean peak titer of 103 (K_v) and the rabbits a mean peak titer of 106 (K_v) comparable to titers of previous preparations, and to those reported in the literature.

The antigenicity of batch 2-96 was also tested in six human volunteers by neutralization and ELISA. The kinetics of the immune response was similar to those of previous preparations and to those reported in the literature (Tables 1, 2) (Figures 1a, 1b, 1c, 2). None of the human volunteers experienced any adverse reactions.

Final Product Composition: The bacteriophage as bottled is suspended in 0.10 M ammonium acetate with 0.001 M CaCl2. Final pH is pH 8.0. The concentration of bacteriophage is 10^{11} pfu/ml. The final protein concentration is 0.09 mg/ml, DNA concentration is 2.48 µg/ml, comparable to previous lots.

Label: Each vial has the following label attached firmly to its outer surface:

> Caution: New drug limited by Federal law to investigational use.
> Bacteriophage ϕX174, 1 x 10^{11} PFU/ml, Lot 2-96
> Hans D. Ochs, University of Washington, Seattle, WA 98195 1.8 (0.8) (2.8) ml

3. Risks and Adverse Reactions:

It is generally accepted that bacteriophage ϕX174 is highly selective in its ability to attach, penetrate and replicate in the host strain, *E. coli C*. We have been unable to find documentation in the literature of bacteriophage producing cytopathic events in human cells. Approximately ten years ago, we had started a collaborative study with Milstein, Walker and Petricciani exploring the possibility of replication of bacteriophage ϕX174 DNA in nonhuman primates, using hybridization with a labeled phage DNA probe. In one of two monkeys, hybridizable ϕX174 DNA sequences were transiently associated with lymphocytes, but not granulocyte DNA; in this one instance, the sequences were present five days after bacteriophage inoculation, but were no longer detectable seven months later. The results suggest that while replication of ϕX174 DNA may occur in primates *in vivo*, the bacteriophage DNA is apparently edited from the host cells.

In reviewing the literature, dating back more than 30 years, no side effects in human volunteers or patients were reported, neither by other investigators who, by published accounts, immunized 76 patients and controls (68-73), nor by investigators using bacteriophage ϕX174 produced in our laboratory (27-67). Excepting the episodes of reactions to Lot 1-88, described below, we have not observed or heard of side effects following immunizations of over 1500 individuals with bacteriophage ϕX174.

In 1988 we produced a new lot (1-88), which was obtained by pooling three batches, each with a relatively low yield of infective particles. This lot was given in 1989 to four volunteers, one of whom reported the onset of "flu-like symptoms", including malaise, chills, and joint and muscle aches, four and one-half hours after a primary phage injection; these symptoms responded to Ibuprofen and resolved completely within 24 hours after immunization. One of the remaining volunteers, after being contacted, reported shortness of breath during the night following primary phage immunization; these symptoms spontaneously resolved the next morning. Subsequently, after contacting all co-investigators who had received Lot 1-88, we identified two patients who recalled symptoms following injection with Lot 1-88. A 35 year old patient with common variable immune deficiency had a very high bacteriophage neutralizing antibody response, limited to IgM, following primary and secondary immunization in 1988 (Lot 1-83); he was immunized a third time, in 1989, to determine if he had developed lasting immunologic memory limited to the production of phage-specific antibody of the IgM isotype after multiple exposure to the antigen. A few hours after receiving Lot 1-88, the patient developed fever, fatigue and arthralgia/myalgia; he was treated by his physician with aspirin and bed rest until disappearance of symptoms 4-6 days after immunization. The patient completely recovered without any sequelae. A second patient, a 10 year old boy with immunodeficiency and atopic symptoms, developed wheezing and shortness of breath seven minutes after his second immunization with Lot 1-88, requiring epinephrine injection and treatment with a nebulizer. He recovered completely within 30 minutes after immunization. Lot 1-88 was recalled by our laboratory. Subsequent lots (7-89; 10-92), used during the last five years, were not associated with reactions.

Review of the literature and our own data indicate that adverse events following immunization with bacteriophage ϕX174 are minor and of low frequency. However, risks as yet unrecognized may exist, and patients receiving this material should be informed of such possibilities prior to consenting to this procedure.

4. Transfer of bacteriophage ϕX174 to other investigators:

It has long been a tradition in our laboratory to make bacteriophage ϕX174 available to co-investigators within the United States and other countries. Until 1994, these other investigators were carried as "co-investigators" if approved by the FDA. Approved co-investigators were able to request bacteriophage ϕ X174 directly from our laboratory, which was then shipped on dry ice. The material was provide free of charge, but at-cost charges were levied for carrying out antibody determinations if the co-investigators requested these. Alternatively, we made available reagents for both the ELISA technique and the neutralization assay system. At the request of the FDA, we have changed this policy in 1994. Each co-investigator will apply for his/her own IND for the use of bacteriophage ϕX174. For safety and product information, the IND holder may refer to the IND (714) provided by Dr. Hans D. Ochs, University of Washington, Seattle, Washington. The bacteriophage ϕX174 will then be shipped to the investigators and administered under the supervision of that investigator.

5. Immunization protocol:

The original protocol for phage immunization was designed in 1972 (27, 32). This protocol has been included in a report by the WHO Committee on Primary Immunodeficiency Diseases, which identifies

bacteriophage ϕX174 as a standard antigen for the assessment of the immune response in man. Effective March 4, 1994, bacteriophage ϕX174 will be made available to investigators outside the state of Washington only if the investigator has obtained an IND from the FDA to use bacteriophage ϕX174 in humans. FDA-approved investigators with documentation will receive bacteriophage ϕX174 of a batch approved by the FDA for use of assessing antibody responses in man. In addition, investigators may request assistance in setting up the neutralization and ELISA techniques in their own laboratories. If the investigator prefers, we will assay each sample at cost for the presence of phage-specific antibody by neutralization or by ELISA.

Bacteriophage ϕX174 is given intravenously at a dose of 0.02 ml/kg body weight using a standard preparation with 1×10^{11} infective phage particles per ml. (27, 32). Blood is obtained before immunization and 15 minutes post primary immunization. Subsequently, a 7 ml aliquot of blood is withdrawn at one week, two weeks, four weeks and six weeks post primary immunization. A secondary immunization is given at six weeks at a similar dose based on body weight. Blood is drawn at one, two and four weeks post secondary immunization. Each serum sample is assayed for antibody by neutralization or by ELISA (58). The one week post primary immunization is routinely checked for the presence of circulating bacteriophage. Phage-specific antibody of the IgG class is determined either by treating the sample with 2 mercaptoethanol (neutralization) (27, 32) or by using specific antisera to differentiate phage-specific IgG and IgM antibody by ELISA (58).

Those patients with an abnormal secondary response consisting of a low proportion of IgG may be selected for a tertiary immunization with bacteriophage to determine if the patient in fact is not capable of switching from IgM to IgG (58). This will have therapeutic implications.

In patients with a "transient" immune deficiency following bone marrow transplantation or chemotherapy, it may be necessary to check the specific antibody response to phage once a year to determine when replacement therapy with IVIG is no longer necessary.

6. Assay systems to measure antibody responses to bacteriophage:

Two techniques are available:

A. ELISA: For this purpose, plates are coated with the same material used for immunization. Serially-diluted sera are added to the plates, incubated, removed, and, after extensive washing, bound human IgG, IgM, or both are identified with the appropriate antibodies. Following extensive washing, individual isotypes of antiphage antibodies or total antiphage antibody are determined using phosphatase-conjugated goat anti-human Ig reagents. The technique is described in detail in Reference 58.

B. Neutralization: Neutralizing antibody activity is measured by a phage-neutralizing assay and the antibody titers expressed as first-order rate constant (Kv) of phage inactivation, as described in detail (27, 32, 58). Upon request the detailed protocol and the reagents for both techniques will be made available to other investigators using material produced in our laboratory.

Normal control values: Normal ELISA units (OD) and graphs indicating $\pm$ 2 S.D. are attached (Figure 1). Table 1 shows normal control values (Kv) and Figure 2 shows a graph for the phage-neutralizing assay system.

7. Bibliographic Information:

The use of bacteriophage ϕX174 as an antigen has been widely studied in both animals and humans. Review of the literature and personal experience indicates that bacteriophage is a potent antigen. Because there is no natural exposure to this antigen, a consistent primary response on first immunization is observed in immunologically normal individuals.

Following a secondary immunization, the antibody response shows amplification and switch from IgM to IgG; approximately 50% of the antibody is of the IgG isotype. Subsequent exposures to the antigen results in a "tertiary" response, showing almost exclusively phage-specific antibody of the IgG class (IgG over 90%). Furthermore, since neither humans nor animals are exposed to this antigen by natural means, no pre-existing antibodies exist and immunoglobulin preparations produced commercially for treatment do not contain phage-neutralizing antibody. Therefore, bacteriophage ϕX174 can be used to measure antibody responses in patients with primary immunodeficiency receiving IVIG infusions. Bacteriophage is particularly useful in identifying patients with X-linked agammaglobulinemia, a majority of whom lack B cells and are unable to mount an antigen-specific antibody; most of these patients are unable to clear bacteriophage ϕX174 immunologically (27, 32). Patients with functional B cells but lack of T help show a characteristic response to bacteriophage characterized by lack of amplification and failure to switch from IgM to IgG (27, 29, 32, 37, 38, 41, 52, 62, 64). Since the assay to assess neutralizing antibody is extremely sensitive, it is possible to detect minute amounts of antibody in patients who produce less than 0.01% of normal antibody responses. Thus, based on the experience obtained by our group and by others, bacteriophage induces a characteristic primary, secondary and tertiary antibody response in immunologically normal controls and characteristic patterns of responses in patients with primary or secondary immune deficiency disorders.

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